CONSEQUENCES OF GONADOTROPIN ADMINISTRATION ON FERTILIZATION AND EARLY EMBRYONIC DEVELOPMENT IN THE DOMESTIC CAT AND THE IN VITRO FERTILIZATION OF FELINE FOLLICULAR OOCYTES

1987

GOODROWE

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
AUG 1987	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Consequences of Gonadotropin Administration on Fertilization and Early Embryonic Development in the Domestic Cat and the In Vitro Fertilization of Feline Follicular Oocytes	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Uniformed Services University Of The Health Sciences Bethesda, MD 20814**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**SAR**	**139**	

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
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Title of Thesis: Consequences of Gonadotropin Administration on Fertilization and Early Embryonic Development in the Domestic Cat and the In Vitro Fertilization of Feline Follicular Oocytes

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Doctor of Philosophy Degree
August 12, 1987

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Committee Chairperson	8-12-87 Date
Committee Member	8-12-87 Date
Committee Member	12 Aug. 1987 Date
Committee Member	8-12-87 Date
Committee Member	8-12-87 Date

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ABSTRACT

Title of Dissertation: Consequences of Gonadotropin Administration on Fertilization and Early Embryonic Development in the Domestic Cat and the *In Vitro* Fertilization of Feline Follicular Oocytes

Karen L. Goodrowe, Doctor of Philosophy, 1987

Dissertation Directed by: David E. Wildt, Adjunct Assistant Professor, Department of Physiology

The purpose of this research was to: 1) investigate effects of an exogenous gonadotropin on embryo recovery, quality and developmental potential in the domestic cat and 2) study factors associated with *in vitro* fertilization of gonadotropin-stimulated domestic and leopard cat follicular oocytes and ejaculated, homologous spermatozoa. In Study 1, females treated with follicle stimulating hormone (2.0 mg FSH-P for 5 days, IE group) and mated produced more corpora lutea and unovulated, cystic-appearing follicles than mated, natural estrus (NE) animals ($P<0.05$). IE cats also produced more ($P<0.01$) poor quality embryos (51.6%) and unfertilized ova (24.4%) than the NE group (21.6% and 2.4%, respectively). Additionally, IE females demonstrated lower circulating estradiol-17ß levels and a premature rise in serum progesterone. Transfer of NE and IE embryos to FSH-P treated recipients resulted in three pregnancies and two litters of kittens. In Study 2, females were treated with pregnant mares' serum gonadotropin (PMSG, 150 IU) followed 72 or 80 hours later with 100 or 200 IU human chorionic gonadotropin (hCG). After laparoscopic collection, follicular oocytes were inseminated with ejaculated, processed spermatozoa, cultured *in vitro* (37°C, 5% $C0_2$) and examined for evidence of fertilization. Selected two- to four-cell stage embryos were transferred to the oviducts of four original oocyte donors. Naturally mated and oocyte donor cats were subjected to laparoscopy and blood sampling to assess corpora lutea function. After 22 to 30 hours of culture, more degenerate oocytes were observed in the 200 (20.6%) than the 100 IU hCG group (8.2%), regardless of the PMSG-hCG interval. Fertilization (48.1%) and cleavage (35.8%) rates were greatest following an 80 hour PMSG-hCG interval combined with 100

IU hCG. Three of four queens receiving embryos became pregnant and produced litters of four, one and three kittens, respectively. Gonadotropin-treated females subjected to follicular aspiration produced morphologically normal corpora lutea and circulating progesterone patterns qualitatively similar ($P>0.05$) to control cats. Leopard cats subjected to the 80 hour PMSG-hCG interval/100 IU hCG treatment developed distinct ovarian follicles and mature oocytes. When cultured with homologous spermatozoa, the fertilization rate was low (14.9%) whereas the number of degenerate oocytes was high (51%).

Viable embryos can be collected and transferred when FSH-P is used to stimulate follicle development, but embryo quality and pregnancy rate after transfer are poor, likely as a result of an altered endocrine environment. Follicular oocytes are capable of fertilization *in vitro*, but success is dependent on both the timing and dose of the hCG stimulus. Follicles subjected to aspiration appear capable of forming normal, functional corpora lutea, and the birth of young after embryo transfer unequivocally demonstrates the developmental competence of *in vitro* fertilized carnivore oocytes.

CONSEQUENCES OF GONADOTROPIN ADMINISTRATION ON FERTILIZATION AND EARLY EMBRYONIC DEVELOPMENT IN THE DOMESTIC CAT AND THE *IN VITRO* FERTILIZATION OF FELINE FOLLICULAR OOCYTES

by

Karen L. Goodrowe

Dissertation submitted to the Faculty of the Department of Physiology
Graduate Program of the Uniformed Services University of the
Health Sciences in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy 1987

ACKNOWLEDGEMENTS

My deepest thanks and gratitude go to Dr. David E. Wildt, my advisor, mentor and friend. His continuous endeavor to reach new peaks of excellence has taught me what it means to be a sound and respected scientist, and has enabled me to pursue my dreams.

To my friends and colleagues, Pat Schmidt, Dr. JoGayle Howard, Dr. Steve Monfort, Mitch Schiewe, Leonard Stuart, Dr. Jack McKenzie, Dr. Lyndsay Phillips, Dr. Mitch Bush, Kathleen James, Shirley Brown, Jan Martenson, Dr. Janine Brown and Annie Miller, I extend my most sincere appreciation for continuous support and cooperation during my graduate education.

I thank Dr. Steve O'Brien, N.C.I., for providing the experimental animals and a continuing interest in carnivore reproduction. I thank Dr. Bob Wall, U.S.D.A., for the use of microscopy equipment and for guidance and advice so freely given.

I thank Dr. Prabir Chakraborty, Dr. Tom Cote, Dr. Joe Dabney, Dr. Greg Mueller and Dr. Jim Terris for their assistance as members of my graduate education committee and for reviewing this manuscript.

I commend the National Zoological Park, the Friends of the National Zoo, the Women's Committee of the Smithsonian and the National Institutes of Health Animal Center Section for their foresight in providing financial, collaborative and technical support to new and innovative research.

Finally, my deepest appreciation and thanks go to my parents, Pat and Ray Goodrowe, for their never ending support, encouragement and belief in my abilities to succeed.

DEDICATION

This research is dedicated to those within the scientific community whose interests lie in the conservation and propagation of endangered species. It is my sincere hope that the information obtained from this project will significantly increase our knowledge of feline reproductive biology and be useful for the procreation of the majestic Felidae family.

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BACKGROUND

The domestic cat is a popular companion animal and is used extensively in biomedical research (see reviews, Meier, 1968; Harrison, 1973; O'Brien, 1986). Because of a close taxonomic and anatomical relatedness to other felid species (Collier and O'Brien, 1985; O'Brien, 1986) and the ease with which it can be handled and maintained under laboratory conditions (Schmidt, 1986), the domestic cat is an excellent model for comparative reproductive studies of the Felidae family.

The behavioral, gonadal and endocrinological events of the estrous cycle of the female domestic cat (queen) are well-documented (see review: Schmidt, 1986). Although laboratory and free-ranging domestic cats reproduce readily, little is known about gamete physiology, developmental biology or artificial breeding. The biological preparations pregnant mares' serum gonadotropin (PMSG) and follicle stimulating hormone (FSH-P) have been tested in the anestrous queen for their effectiveness in eliciting follicular development and estrual behavior (Wildt, 1986). However, most efforts have been directed at simple observational studies of estrus/ovulation induction rather than investigating the integrative impact of these preparations on physiological function. One study reported the comparative responsiveness of the cat ovary to varying dosages of PMSG versus FSH-P (Wildt et al., 1978b). From these data, it is apparent that the gonads of the female cat are highly sensitive to these specific exogenous gonadotropins; if the drug dosage is extreme or the administration interval prolonged, persistent follicular cysts form which can have pathologic consequences.

Although kittens have been produced after artificially inseminating FSH-P-treated queens (Platz et al., 1978), virtually nothing is known about the physiological consequences of gonadotropin treatment in terms of normalcy of the estrous cycle, gestation, oocyte/embryo viability, sperm transport or sperm/oocyte interactions during fertilization. Successful reproduction in cats is dependent on a comprehensive

understanding of normal reproductive events as well as the physiological alterations induced by exogenous hormonal therapy.

For the domestic cat, the techniques of embryo transfer and *in vitro* fertilization/embryo transfer are two powerful approaches permitting the detailed study of developmental biology. Additionally, basic technology developed in the domestic cat offers exciting possibilities for improving the reproductive potential of nondomestic species of Felidae. Embryo transfer is the process of placing pre-implantation stage embryos collected from the reproductive tract of one female (the donor) into the uterus or oviducts of one or more other females (the recipients) to complete gestation. The resulting offspring are genetically related to the original donor rather than the surrogate mother. Generally, to stimulate follicular maturation and produce a greater than normal number of released oocytes at ovulation, exogenous gonadotropins are given to the donor female. *In vitro* fertilization (IVF) is the union of spermatozoa and ova outside the female reproductive tract, usually in a controlled, species-specific culture environment. Generally, oocytes are collected from a hormonally-stimulated female by either flushing the oviducts or aspirating directly from ovarian follicles. After fertilization, the zygotes are transferred back to the original oocyte donor (autotransfer) or to genetically unrelated, surrogate females.

Embryo studies, when conducted in the context of basic research, laboratory animal management or the commercial improvement of human/animal reproduction, have provided a wealth of information on the processes of ovulation, fertilization, early embryonic development and implantation as well as the feasibility of gamete/embryo cryopreservation and embryo micromanipulation. In Western countries, livestock embryo transfer is a major industry. In 1972, a total of 20 offspring were produced in the United States from embryo transfers in cattle whereas over 50,000 calves were produced in 1985 (Adams, 1985). Embryo transfer in monotocous, genetically superior individuals, maximizes reproductive efficiency because females can be hormonally-stimulated to produce more embryos in a single collection than normally would result in offspring in a lifetime. Transfer of fertilized

ova to less valuable recipients frees the donor from being encumbered by pregnancy and permits the embryo collection process to be repeated.

In vitro fertilization plays a major role in alleviating human infertility. The birth of Louise Brown in 1978 (Steptoe and Edwards, 1978), the first human born as a result of *in vitro* fertilization, revolutionized medical concepts for circumventing problems associated with damaged or blocked oviducts, poor sperm transport through the cervix, oligospermia, teratozoospermia and immunological reactions of the female to seminal fluid (Blasco and Tureck, 1985; Garcia, 1985). Since 1978, more than 2,000 babies have been born as a result of IVF (Jones, 1984; Blasco and Tureck, 1985; Garcia, 1985; Marrs, 1986). For animals, *in vitro* fertilization is useful for improving our understanding of the fertilization process while offering a number of potential advantages for propagation. Domestic as well as nondomestic species frequently are behaviorally incompatible making natural mating impossible. Physical handicaps or age often can preclude the natural copulatory event, and many mammalian species, like humans, experience infertility of unknown etiology (Wildt, 1988). With rare species, the application of IVF to propagation programs provides two possible benefits. First, many species are aggressive, highly temperamental and difficult to physically transport between breeding facilities. The shipment of spermatozoa, oocytes and/or embryos is less risky to the species than physical transport of the animal. Secondly, many rare mammalian species exist in limited-sized populations which often result in increased genetic homozygosity which appears to directly or indirectly contribute to impaired reproductive function (O'Brien et al., 1983, 1985; Wildt et al., 1986a). The use of IVF and related techniques such as gamete/embryo cryopreservation would allow developing an ancillary strategy for the controlled management of these small populations. The laboratory fertilization of gametes either collected from geographically diverse individuals or thawed after years of cryo-storage could be an effective approach for re-introducing or sustaining biological diversity within endangered species populations.

With the exception of the carnivores, the physiological factors affecting the success of embryo recovery, transfer and IVF have been studied extensively in a diverse selection of laboratory and farm animal species. The paucity of literature involving carnivores is due primarily to two factors: 1) these animals do not serve as a human food source and thus, there is little financial incentive to improve reproductive efficiency; and 2) compared to many species used in developmental biology research (i.e., mice, rats, hamsters), carnivores generally are more expensive to purchase and maintain under laboratory conditions. As a result, the number of scientific publications concerning the reproductive biology of the order Carnivora and specifically of species within the Felidae family is relatively scarce. There are similarities as well as species-specific physiological, anatomical and genetic traits among the carnivores. Because the data base is severely limited, it is relevant to review pertinent literature from all species within the order.

ESTRUS INDUCTION

Considerable information is available concerning the use of exogenous gonadotropins for inducing follicular development, estrous behavior and ovulation in a variety of laboratory and farm animal species. For these purposes, the most common, commercially available hormones include FSH-P, PMSG and human chorionic gonadotropin (hCG). The anterior pituitary protein, follicle stimulating hormone is a glycoprotein containing approximately 25% carbohydrate and composed of two dissimilar subunits (α and ß, the latter being responsible for the biological specificity), each with a molecular weight of approximately 16,000. For commercial use, this hormone is acquired by purifying porcine pituitary extracts, resulting in preparations with predominantly FSH activity but also some luteinizing hormone (LH) contamination (FSH:LH ratio range, 0.2 to 4.5, Armstrong and Opavsky, 1986; Lindsell et al., 1986). The gonadotropins PMSG and hCG also are high molecular weight glycoproteins (28,000 to 53,000 and 37,800,

respectively) containing 30 to 50% carbohydrate, a large portion of which is sialic acid. The α subunits of LH, FSH, hCG and PMSG are similar in composition. Because of the comparable biochemistry and biological activity of the ß subunits, hCG often is used as a substitute for LH to artificially induce ovulation. During the first trimester of human pregnancy the placental trophoblastic cells produce hCG which is responsible for corpus luteum (CL) maintenance. During the early stages of the mare's pregnancy, the maternal endometrial cup cells secrete PMSG which has similar biological properties to endogenous follicle stimulating hormone. The plasma half life of these compounds varies markedly (LH: 30 minutes, FSH: 149 minutes, hCG: 4.9 hours and PMSG: 26 hours; Sherwood and McShan, 1977); thus when administered exogenously, multiple injections of exogenous LH and FSH are required to elicit similar responses obtained with only a single injection of hCG or PMSG. These preparations have been used in only a few carnivore studies (generally in the cat and dog), and to date a suitable gonadotropin regimen for consistently inducing follicular maturation without ovarian hyperstimulation has yet to be established.

Canine

The bitch is seasonally monoestrous, ovulating spontaneously during the first 5 days of natural estrus (Schwartz, 1977). The literature suggests that hormonal induction of follicular activity in the dog is difficult with most attempts being minimally or inconsistently successful. In an early study, Scrogie (1939) reported that a single subcutaneous injection of PMSG and human pregnancy urine induced estrus in 14 of 18 anestrous females 2 to 6 days after treatment. Estrus induction also has been reported from using repeated intramuscular injections of PMSG (Thun et al., 1977). Dosages of 250 to 500 IU PMSG once/day for 10 days or 20 IU PMSG/kg for 10 days in combination with 500 IU hCG elicited estrous behavior and ovulation in 14 of 25 treated bitches.

Archbald et al. (1980) reported that 44 IU PMSG/kg given intramuscularly for 9 days followed with 500 IU hCG on Day 10 induced estrous behavior on Days 5 through Day 19 in five of five treated females. However, the same regimen given as a subcutaneous injection elicited sexual receptivity and ovulation in only two of 25 and four of 25 treated bitches, respectively. In a related study involving a total of three weekly intramuscular injections of PMSG (100 IU/kg), eight of 11 animals exhibited estrous behavior while 10 of 11 ovulated (Wright, 1980). Corpora lutea numbers ranged from two to 60/animal (mean, 18.5 CL).

Pre-treatment of females with estrogens alone or in combination with PMSG (200 to 400 IU) and hCG (1000 IU) also induces estrous behavior and ovulation in dogs (Takeishi et al., 1976). Six of seven bitches treated with PMSG and hCG administered intramuscularly on the day of withdrawal bleeding (proestrus) and again on Day 1 of estrus became pregnant and delivered puppies after a series of natural matings or artificial inseminations. Chakraborty et al. (1982) indicated that a 200 μg dose of estradiol given intramuscularly every other day for 3 days could produce a proestrous discharge 4 to 7 days later. These investigators also demonstrated that PMSG (500 or 1000 IU) in combination with hCG (500 or 1000 IU) given intramuscularly on the first day of bleeding and again 6 days later produced sexual receptivity and ovulation in 80% and 60% of the animals treated, respectively. However, commercially available FSH-P (5 mg/day, seven to 10 days) was ineffective in promoting follicular development.

Feline

The domestic cat is seasonally polyestrous, ovulating only after cervical stimulation is induced by coitus or a comparable artificial stimulus (Greulich, 1934; Dawson and Friedgood, 1940). The length of the estrous cycle in cats averages 14 to 21 days (range, 6 to 21 days; McDonald, 1975; Jemmett and Evans, 1977; Wildt et al., 1978a). The duration of behavioral estrus of the free-ranging queen is 6 to 10 days (Scott and Lloyd-Jacobs,

1955) which corresponds with a mean length of 5.8 days for the laboratory-maintained animal (Wildt et al., 1978a).

Because the cat is an induced ovulator (Schwartz, 1977), a rise in serum luteinizing hormone (LH) occurs only after mating or a comparable cervical stimulus. The magnitude of the LH surge and the frequency of ovulation are proportionally related. The rise in LH is less pronounced and ovulation occurs less frequently when only a single copulatory stimulus is provided (Concannon et al., 1980; Wildt et al., 1980). Repeated matings on a single or on consecutive days of estrus stimulate more pronounced LH surges and a greater incidence of ovulation. A refractoriness in the ability of the pituitary to release LH in response to repeated matings occurs with a gradual decline in circulating LH observed as matings occur over time (Concannon et al., 1980; Wildt et al., 1980; Johnson and Gay, 1981; Wildt et al., 1981a; Banks and Stabenfeldt, 1982; Shille et al., 1983; Glover et al., 1985).

The coitus-to-ovulation interval in cats probably is related to the number of and time between matings. Wildt et al. (1981a) allowed queens to mate three times/day at 3 hour intervals for 3 days. In five of 12 cats, ovulation occurred between 48 and 52 hours after the mating-induced LH surge (96 to 100 hours after the first copulation) while follicular rupture did not occur in seven animals until 52 or more hours after the LH peak. Shille et al. (1983) allowed *ad libitum* matings within a single 30 minute period and observed ovulation 24 to 32 hours later. The latter results are similar to the onset of ovulation after an exogenous, LH-type hormonal treatment, ovulation occurring 24 to 30 hours following an hCG injection (Hamner et al., 1970; Bowen, 1977; Niwa et al., 1985).

Queens experiencing a natural estrus ovulate in response to hCG or gonadotropin releasing hormone (GnRH). Single intramuscular injections of 50, 100, 250 or 500 IU of hCG on Day 1 of estrus result in a mean ovulation rate (% mature ovarian follicles ovulating) of 32, 25, 67 and 100%, respectively (Wildt and Seager, 1978). Single injections of the same doses on the first and second days of estrus change the ovulation

rates to 42, 74, 85 and 96%, respectively. However, all animals receiving two injections of hCG at the 100, 250 or 500 IU level elicit an ovulatory response. A 250 IU dose of hCG given intramuscularly on the first and second days of mating produces a mean of 4.1 CL/female (Goodrowe and Wildt, 1987).

Female cats receiving Pergonal (a hormonal preparation containing the equivalent of 75 IU FSH and 75 IU LH, Serono Laboratories, Inc., Braintree, MA) ovulate following a single intramuscular bolus of hCG (Kam, 1981). A dose of 60 IU of hCG given 5 days after Pergonal causes 60% of the treated females to ovulate, whereas an 80 IU dose elicits an ovulatory response in all treated animals. However, these data are presented as the percentage of females ovulating and are not representative of the proportion of follicles rupturing.

Treating cats with 25 μg GnRH (intramuscularly) on the second day of estrus induced ovulation in four of four cats with 100% of the follicles rupturing (Chakraborty et al., 1979). Goodrowe and Wildt (1987) noted that this same dosage given on the second and third days of estrus produced a mean of 4.0 CL and 0.9 unovulated follicles. When GnRH is administered in combination with coital stimuli, ovulation is not enhanced. In contrast, when mating (three times/day at 3 hour intervals for the first 3 days of estrus) and hCG (250 IU intramuscularly on Days 2 and 3 of estrus) are combined, a two-fold increase in CL number is observed in both natural estrus and FSH-P-treated cats (Goodrowe and Wildt, 1987). This enhanced ovulation rate does not decrease the number of cystic-appearing, anovulatory follicles. In natural estrus cats, the combination of coitus and hCG results in additional, anovulatory follicles (five-fold increase), a finding which is not observed when either exogenous stimulus is applied alone. This action likely is a result of hCG synergizing with a mating-induced pituitary release of FSH.

Crude pituitary extracts of FSH and LH were used as early as 1935 to induce estrus, follicular development and ovulation in the domestic cat (Foster and Hisaw, 1935). After treating anestrous females with follicle stimulating hormone extract (2 g

subcutaneously for 5 days), a mean ovulation rate of 15 CL was observed in 11 of 15 females. Administering FSH at the same dosage in combination with 0.1% LH for 5 days increased the mean ovulation rate to 20 CL. Further increasing the LH concentration of the mixture to 1 to 4% tended to inhibit follicular rupture. Administering crude human pregnancy urine or pregnant mares' serum extracts to domestic cats also elicits estrous behavior (Windle, 1939). Of 40 queens receiving urine extracts, 31 exhibited sexual receptivity and allowed copulation, but only 16 became pregnant. A total of 69 females divided into two groups (estrus and anestrus) received pregnant mares' serum extracts. When anestrous females (n=37) received serum extracts, most (n=34) copulated but only 47% became pregnant. Thirty-one of 32 estrous females treated with serum extracts mated and of these, 20 exhibited follicular growth, ovulated and/or became pregnant.

The first use of a partially purified gonadotropin for stimulating ovulation in cats was reported by Colby (1970) who gave an 8 day series of intramuscular PMSG injections (total dose range, 250 to 500 IU). Although pregnancies resulted from mating hormonally-treated females, only a few cats were allowed to carry kittens to term. Specific data on the proportion of females becoming pregnant following mating were not provided. The gestation interval in PMSG-treated cats was 3 to 7 days greater than the average 63 day interval for untreated domestic cats. Administering PMSG to females less than one year of age resulted in an ovarian hyperstimulation consisting of a superovulatory response (as many as 60 CL/cat) and the presence of cystic-appearing, anovulatory follicles (1 to 5 mm in diameter).

Similar responsiveness was observed by Wildt et al. (1978b) in a comparative study of the ovarian activity of cats given PMSG versus FSH-P. When PMSG was given either as a single intramuscular bolus (1000 IU) or over an 8 day period (500 IU total), relatively few ovulations were observed (3.7, 8.0; respectively); however, large numbers of unovulated, cystic-appearing follicles were detected (6.3, 10.2; respectively). When FSH-P was administered as a single injection (10 or 15 mg intramuscularly), only four of

12 treated animals exhibited estrous behavior, although follicular activity was observed laparoscopically in ten females. This same gonadotropin, given in daily intramuscular doses of 1, 2 or 3 mg for 7 days, induced estrus and ovulation in all treated animals. The 2 mg dose was the most satisfactory, producing the greatest number of CL (10.3) and the lowest number of cystic-appearing, anovulatory follicles (7.3). In a subsequent study, five of seven animals receiving this gonadotropin regimen and allowed to mate, conceived and delivered normal offspring. Pregnancies also resulted from artificially inseminating females with frozen-thawed spermatozoa following treatment with FSH-P (2 mg/day for 5 days; Platz et al., 1978). However, conception rates were low (10.6%).

Administering PMSG in a single bolus (100 IU intramuscularly) to anestrous cats followed 5 to 7 days later by an injection of hCG (50 IU intramuscularly) produced ovulation and pregnancy results comparable to natural matings (Cline et al., 1980). However, daily injections over a 4 to 5 day period resulted in fewer pregnancies and a decrease in the survivability of kittens to weaning. As observed in earlier studies (Colby, 1970; Wildt et al., 1978b), the higher doses of PMSG resulted in ovarian hyperstimulation with the production of cystic-appearing, anovulatory follicles.

Although administering gonadotropins has been successful in eliciting follicular development and ovulation in a number of other Felidae species (Moore et al., 1981; Wildt et al., 1981b; Dresser et al., 1982; Phillips et al., 1982), subsequent pregnancies have occurred only on two occasions (Moore et al., 1981; Dresser et al., 1982) and the results have not been repeatable. Moore et al. (1981) induced follicular maturation and ovulation in three pumas (*Felis concolor*) using PMSG (1250 IU intramuscularly) followed 72 hours later with hCG (1000 IU intramuscularly). Fresh spermatozoa collected by electroejaculation were washed with Tyrode's solution (TCS, Tyrode, Difco Laboratories, Detroit, MI) and placed into the uterine horns (at laparotomy) of all hormonally-treated females. A single conception with the birth of one cub resulted. Dresser et al. (1982) successfully induced ovulation in a naturally estrous, Persian leopard (*Panthera pardus*

saxicolor) with two intramuscular injections of hCG administered at a 20 hour interval. Fresh spermatozoa (1.5×10^8), collected by electroejaculation, were deposited into the uterus (via the cervix) 20 hours after each hCG injection. After a gestation of 96 days a single, still-born cub was delivered.

Treatment with FSH-P (10 mg intramuscularly for 5 consecutive days) has been relatively successful for inducing ovulation in the cheetah (*Acinonyx jubatus*), lion (*Panthera leo*) and North Chinese leopard (*Panthera pardus japonensis*). In contrast, the female tiger (*Panthera tigris*), treated with the same FSH-P dose usually develops swollen, hyperemic ovaries with immature follicles (Wildt et al., 1987). Uterine and oviductal flushes of FSH-P-treated tigresses 1 hour after artificial insemination contained no spermatozoa (Wildt et al., 1987) indicating impaired spermatozoal transport. Although 26 of 30 female cheetahs treated with FSH-P demonstrated follicular development, only one of 30 exhibited estrous behavior and none of those inseminated with fresh or thawed spermatozoa became pregnant (Wildt et al., 1986b). Collectively, these data indicate that the feline ovary is extremely sensitive to exogenous gonadotropins. It is possible that the environment of the female's reproductive tract is compromised by an altered or aberrant endocrine milieu, thereby adversely affecting sperm transport, fertilization or early embryonic development. This hypothesis is supported by studies of other species in which exogenous gonadotropin treatment or the presence of cystic follicles results in poor sperm transport (Evans and Armstrong, 1984b), oocyte/embryo abnormalities (Fujimoto et al., 1974; Maudlin and Fraser, 1977; Moor et al., 1985) or a reduced fertilization rate (Evans and Armstrong, 1984a).

EMBRYO RECOVERY AND TRANSFER

The recovery of mammalian oocytes was first described by de Graaf in 1672 when ova were recovered from a rabbit reproductive tract 72 hours post-coitus. The first

successful transfer of mammalian embryos from donor to recipient with subsequent birth of live offspring was accomplished in the rabbit by Heape in 1891. Embryo transfer studies, mostly conducted within the last 35 years have produced offspring in 19 species (Table 1). The first successful embryo transfer in the cat and dog occurred in 1979, and in both cases, embryos were transferred between animals which had experienced a recent natural estrus. To date, embryo transfer with the birth of live-young has not been reported in any carnivore species after treating the donor or recipient with exogenous gonadotropins.

Canids

The mechanisms associated with ovulation, oocyte maturation, oviductal transport and early embryonic development in the domestic dog have been discussed and documented by a number of investigators (Bischoff, 1845; Evans and Cole, 1931; Whitney, 1940; Gier, 1950; Holst and Phemister, 1971; Phemister et al., 1973). Holst and Phemister (1971) flushed the reproductive tracts of 41 bitches 2 to 21 days after breeding and collected embryos ranging from unfertilized ova to morulae (5 and 12 days post-coitus) and early to expanded blastocysts (8 to 11 days post-breeding). The discrepancy in embryonic development rates probably resulted from differences in the timing of ovulation and fertilization with respect to the onset of estrus and mating.

The only report of embryo collection and transfer in the dog was described by Kinney et al. (1979). Nine to 10 days after the first mating, the uterine horns of naturally estrous bitches were flushed at laparotomy with Ham's F-10 medium supplemented with 25 mM Hepes buffer and 10% heat-treated fetal calf serum. A total of 72 embryos was recovered from 26 collection attempts. After transferring 37 embryos to seven naturally synchronized recipients, three pregnancies were diagnosed and a total of four pups born.

Table 1. First successful embryo transfer by species

Date	Species	Investigator
1891	Rabbit	Heape
1933	Rat	Nicholas
1935	Goat	Warwick et al.
1942	Mouse	Fekete and Little
1949	Sheep	Warwick et al.
1951	Cow	Willet et al.
1951	Pig	Kvasnickii
1963	Quokka	Tyndale-Biscoe
1964	Golden hamster	Blaha
1968	Ferret	Chang
1970	Tammar wallaby	Tyndale-Biscoe
1974	Horse	Oguri and Tsutsumi
1976	Baboon	Kraemer et al.
1977	Rhesus monkey	Marston et al.
1978	Mink	Zheleznova and Golubitsa
1978	Human	Steptoe and Edwards
1979	Dog	Kinney et al.
1979	Cat	Kraemer et al.
1981	Mongolian gerbil	Norris

Embryos also have been collected from bitches induced into estrus with PMSG (44 IU/kg, intramuscularly) given daily for 9 days (Archbald et al., 1980). Eight to 12 days after the first mating, the reproductive tracts were flushed at laparotomy with phosphate buffered saline supplemented with 3% bovine serum albumin (a combination referred to as PB1 medium, Whittingham, 1971). A total of 78 embryos was collected from six of 10 animals. The stage of embryonic development ranged from an unfertilized ova to early morulae with a recovery rate of 75% (embryos collected/number of CL observed). In contrast to the findings of Holst and Phemister (1971), no blastocysts were collected, even as late as Day 12, suggesting a gonadotropin-induced delay in embryonic development.

One embryo-related investigation has been conducted in a nondomestic canid, the silver fox (Pearson and Enders, 1943). By taking tissue sections at autopsy from 33 females at various times post-coitus, the processes of oocyte maturation, fertilization, early embryonic development and transport were studied. These authors concluded that the mechanisms of ovulation, CL formation, polar body extrusion and early embryonic development are similar between the fox and dog.

Mustelids

Artificial insemination and subsequent embryo collection in the common ferret were accomplished by Chang and Yanagimachi (1963). Epididymal spermatozoa, collected after castration, were surgically placed into the ovarian capsule of naturally estrual females at various intervals before and after ovulation. A total of 278 embryos (one- to four-cells) and/or unfertilized ova was collected from 47 dissected oviducts 1.5 to 78 hours after ovulation. To develop a fertilizing capacity (capacitation), ferret spermatozoa required 3.5 to 11.5 hours of residence in the female reproductive tract. The proportion of penetrated oocytes (54 to 62%) did not differ when insemination into the ovarian capsule occurred from 6 hours before to 12 hours after ovulation. However, when spermatozoa were

deposited 24 to 36 hours after ovulation, fertilization rates were decreased significantly (14 to 30%).

Embryos also have been collected from naturally estrous ferrets induced to ovulate with 90 IU hCG given intraperitoneally (Chang, 1965). Intra-uterine inseminations with epididymal spermatozoa were performed at various intervals following gonadotropin injection. Fertilization rates were highest (73%) when insemination occurred at the time of hCG injection and decreased as the ovulation induction-to-insemination interval increased.

Embryo transfer in the ferret with subsequent fetal development also was described by Chang (1968). Fifty-one morula to blastocyst-stage embryos were transferred to the uterine horns of hCG-treated, naturally estrous recipients 6 to 8 days after the donors were mated. Implantation was observed in all eight recipients, even in asynchronous transfers (Day 8 embryos to Day 6 uteri and Day 6 embryos to Day 8 uterine horns). One female allowed to gestate to term delivered four live-young.

Although Chang (1968) had no success in transferring 11 mink embryos to two mink recipients, Zheleznova and Golubitsa (1978) reported a 25% pregnancy rate after transferring blastocyst-stage embryos between the uteri of suitably synchronized mink.

Heterologous gamete interaction also has been studied in mustelids. Chang (1968) investigated the possibility of interspecific insemination and embryo transfer between the ferret and mink (a delayed implanter). Although mink spermatozoa were capable of fertilizing ferret oocytes with low rates of implantation, the reciprocal experiment failed. A similar phenomenon occurred when interspecies embryo transfers were performed between the ferret and mink. Ferret embryos transferred to the mink uterus survived for only 6 to 10 days, degenerating without implantation. Conversely, mink embryos were capable of undergoing implantation when transferred to the ferret uterus, but degenerated 4 to 8 days after penetration of the endometrium.

The development of ferret embryos in the rabbit oviduct and uterus also has been investigated (Chang 1966). A 2 day interval in the rabbit oviduct supported embryonic

growth; however, after flushing on the fourth day, no embryos were recovered. The rabbit uterus proved to be an unsuitable culture environment. A subsequent study demonstrated that the culture of ferret embryos in the rabbit oviduct severely compromised further developmental capacity (Chang et al., 1971).

Felids

There are few embryo-related investigations of the domestic cat. Herron and Sis (1974) flushed embryos from excised tracts of naturally estrous females pre-treated with saline or estrogen for 3 or 6 days post-coitus (Days 3 to 6). Nineteen eight-cell embryos were collected on Day 3 from saline-treated females while only one degenerate ovum was found on Day 6. All embryos collected from estrogen-treated females on Day 6 were degenerate and located in the oviducts rather than the uterine horns, indicating treatment-delayed transport.

The literature contains a single account of embryo collection and transfer in the domestic cat (Kraemer et al., 1979), a study accomplished using donors and recipients in natural estrus. Donor queens were allowed multiple matings for 1 or 2 days while recipients were induced to ovulate by sterile matings with a vasectomized male or by two intramuscular injections of hCG (250 IU/day on the first 2 days of estrus). Uterine horn flushes using Ham's F-10 medium containing 25 mM Hepes buffer (50 ml) were performed by laparotomy 6, 7 or 9 days after mating. Forty-seven embryos, collected during 17 flush attempts, were transferred in Ham's F-10 or Ham's F-10 supplemented with fetal calf serum to the uteri of nine recipients. Four pregnancies resulted, three from queens mated with a vasectomized male. Three litters of kittens were born.

In a preliminary study, Kraemer (1983) reported the collection of three morulae from an African lioness during five attempts. The embryos were frozen and thawed, but no pregnancy resulted after autotransfer.

Photographic documentation of feline embryos in the literature is limited, while the morphologic description of unfixed, unstained cat oocytes has yet to be reported. Bowen (1977) published the only photographs of feline embryos which indicates that blastomeres at all stages of development (two-cell to blastocyst) maintain a dark, homogeneously opaque appearance. This characteristic, attributable to a high concentration of dark, dense lipid within the cytoplasm (Guraya, 1965), is commonly observed in pig, dog and ferret ova and embryos.

IN VITRO FERTILIZATION

Although fertilization *in vitro* has been reported for a number of species (see reviews: Rogers, 1978; Wright and Bondioli, 1981; Brackett, 1985), successful IVF with subsequent birth of live-offspring has been accomplished only in the rabbit (Chang, 1959), mouse (Whittingham, 1968), rat (Toyoda and Chang, 1974), human (Steptoe and Edwards, 1978), cow (Brackett et al., 1982), three species of non-human primates (Clayton and Kuehl, 1984; Bavister et al., 1984; Balmaceda et al., 1984) and most recently the pig and sheep (Cheng et al., 1986). Despite continuous IVF research, offspring have been consistently produced only in the mouse, rat and human with the latter now commonly serving as a model system for developing technology in many other mammalian species.

The major factors influencing the success of IVF are: 1) maturity of the retrieved oocyte; 2) spermatozoal quality, number and capacitation; 3) culture environment and components; and 4) synchronization of the recipient female.

Oocyte Maturity

Maturation of the oocyte is a complex event rendering the gamete capable of fertilization and subsequent normal development. Alterations in the oocyte nucleus,

cytoplasm, plasma membrane and the surrounding cumulus oophorus cells all are prerequisites to fertilization (Brackett, 1985; Motlik and Fulka, 1986).

Nuclear maturation. At puberty, female gametes are in a stage of meiotic arrest in the dictyate phase of prophase I. This quiescent stage apparently is controlled by an intrafollicular oocyte maturation inhibitor (Chang, 1955; Foote and Thibault, 1969; Channing et al., 1982; Moor et al., 1983) which loses its effect in response to a gonadotropic stimulus (Moor et al., 1983). In response to the LH surge or exogenous hCG, meiosis is resumed, resulting in germinal vesicle breakdown (GVBD) and extrusion of the first polar body, all occurring immediately before the ovulatory event in most species. These nuclear maturation events proceed through metaphase II at which time nuclear division again becomes arrested. Re-activation of the oocyte chromosomal material is initiated by spermatozoal penetration of the ooplasm resulting in the extrusion of the second polar body and the formation of the female pronucleus.

Cytoplasmic and plasma membrane maturation. Maturation of the cytoplasm remains largely unstudied. Changes in RNA content and protein synthesis occur in mammalian oocytes during follicular growth (Moor et al., 1983; Wassarman, 1983; Motlik and Fulka, 1986) providing the female gamete with a reservoir of supportive nutrients necessary for early embryonic development.

Disrupting the communication between the corona radiata cells and the plasma membrane (oolema) near the time of ovulation may be necessary for cortical granule alignment adjacent to the cytoplasmic membrane (Sathananthan and Trounson, 1982; Schnell et al., 1983). These cellular components then serve to prevent polyspermy (numerous sperm cells penetrating the oolema) during fertilization. Other changes in the ooplasm include a depolarization attributed to an increased potassium permeability (Biggers et al., 1977) and increased amino acid transport from the cells of the corona radiata into the ooplasm following GVBD (Moor and Smith, 1978).

Changes in the cumulus-oocyte complex. Oocyte maturation is dependent upon a direct communication between the granulosa (cumulus oophorus) cells and the oocyte (Moor and Trounson, 1977; Gilula et al., 1978; Crosby et al., 1981; Dekel et al., 1981; Moor et al., 1981; Eppig, 1982; Moor et al., 1983; Motlik et al., 1986). Microvilli from the granulosa cells are extended to the germ cell and joined to it by gap junctions at the oocyte membrane. These intercellular communications are responsible for supplying the ovum with nutrients and hormonal stimuli necessary for growth and maturation. Although sheep and mouse oocytes devoid of cumulus cells are capable of nuclear maturation, normal embryonic development will not proceed in culture. In contrast, intact sheep cumulus-oocyte complexes matured and exposed to LH *in vitro* have the potential for further embryonic development (32% culture to the blastocyst stage, Crosby et al., 1981). Oocytes obtained from intact sheep follicles matured *in vitro* and exposed to gonadotropins also have a high rate of development following fertilization (over 50% culturing to the blastocyst stage, Moor et al., 1981, 1983), demonstrating that these intracellular communications are vital for achieving full developmental capacity.

Morphological alterations in the cumulus-oocyte complex (indicative of maturation) are observed readily by stereomicroscopy. In a preovulatory follicle or in an *in vitro* culture system, the corona radiata and cumulus oophorus are tightly compacted around the ovum. The endogenous LH surge or administration of exogenous hCG is followed by a reduction in cumulus cell-oocyte coupling and the initiation of germinal vesicle breakdown (Gilula et al., 1978; Moor et al., 1981; Eppig, 1982; Motlik et al., 1986). Morphologically, this event is characterized by an expansion or "loosening," first of the cumulus oophorus cells and, approximately 8 hours later, by the corona radiata cells. At the time of ovulation, cumulus cell expansion is pronounced and the intracellular coupling between the corona radiata cells and the oocyte is diminished. Fertilization rates *in vitro* have been positively correlated with the degree of cumulus and corona expansion (Testart et

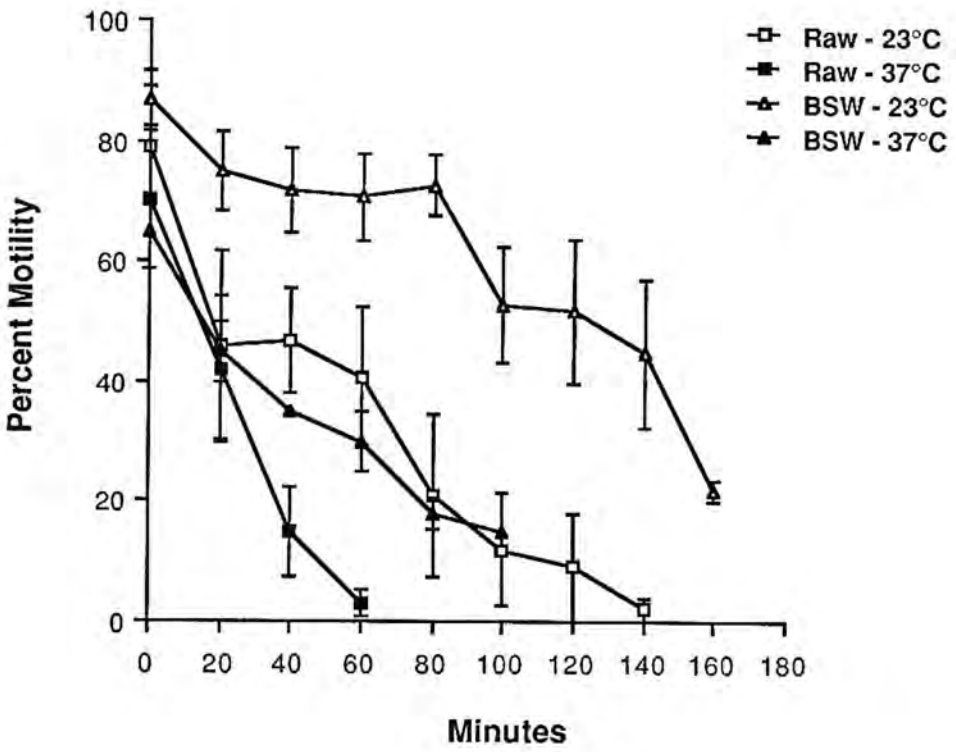
al., 1983), and human IVF clinics now commonly use these variables as subjective estimates of oocyte maturity.

Spermatozoa

Ejaculate quality. Spermatozoal quality influences the efficiency of an *in vitro* fertilization system. In the human, spermatozoal motility ratings less than 30 to 50% have been directly correlated with decreased oocyte penetration (Testart et al., 1983; Mahadevan and Trounson, 1984; Kruger et al., 1986). Teratozoospermia (a high proportion of morphologically abnormal spermatozoa) and oligospermia (fewer than normal sperm cells/ejaculate) also have been associated with reduced IVF rates (Testart et al., 1983; Mahadevan and Trounson, 1984). Concentration of the spermatozoa in the fertilization medium also influences the rate of fertilization with fewer spermatozoa (in the 10^4 to 10^5 range) being preferable in the mouse, rat and human because greater concentrations can lead to polyspermia (Fraser and Maudlin, 1979; Nishimura et al., 1982).

Maintenance of spermatozoal viability *in vitro*. Conventional methods for collecting and processing livestock semen generally involve maintaining the spermatozoa at 37°C, a factor apparently critical to preventing cold shock of the cells (Salisbury and Willet, 1940; MacMillan et al., 1966; Salisbury et al., 1978). The literature contains little information on promoting *in vitro* viability of felid spermatozoa. In a preliminary report from this laboratory, we noted only a short duration of *in vitro* motility (less than 3 hours) for undiluted domestic cat spermatozoa (37°C)(Fig. 1). Suspension of the sperm cells in a modified Biggers' Stern and Whittingham (mBSW) culture medium (Bowen, 1977) significantly increased longevity, and washing of the sperm cells to remove seminal fluid prolonged duration of viability even further.

Figure 1. Duration of electroejaculated domestic cat spermatozoal motility *in vitro*. Ejaculates, which were examined every 20 minutes, were assigned to one of four treatments (n=4/treatment) and maintained: 1) undiluted at 23°C (□), 2) undiluted at 37°C (■), 3) diluted 1:1 with mBSW medium at 23°C (Δ) or 4) diluted 1:1 with mBSW medium at 37°C (▲). Values are means ± S.E.M.


Raw - 23°C
Raw - 37°C
BSW - 23°C
BSW - 37°C
Percent Motility
100
80
60
40
20
0
0
20
40
60
80
100
120
140
160
180
Minutes

Capacitation. The phenomenon of capacitation, originally described by Austin (1951) and Chang (1951), involves a series of spermatozoal changes necessary for fertilization. Capacitation is complex, consisting of many undefined events occurring within the sperm plasma membrane which prepare the cell for the acrosome reaction. In the latter, enzymes are released from the acrosomal region of the spermatozoon which facilitate penetration of the oocyte's cumulus cell mass and zona pellucida (Yanagimachi, 1981). Yanagimachi (1981) and Clegg (1983) have reviewed the current theories associated with capacitation mechanisms. The major factors inducing the acrosome reaction are: 1) an increased permeability of the sperm plasma membrane to calcium; 2) a modification of membrane structure including removal, addition and/or movement of surface and intra-membrane components, all of which contribute to the formation of specialized fusigenic areas; 3) an activation of adenylate cyclase and increased intracellular cyclic-AMP to phosphorylate proteins necessary to increase motility, thereby causing an increase in spermatozoal metabolic rate and respiration; and 4) a conversion of the enzyme pro-acrosin to acrosin, necessary for penetration of the zona pellucida by the spermatozoon. Although the definitive processes associated with capacitation remain unresolved, it is clear that fertilization cannot occur without the sperm cell experiencing this phenomenon (Yanagimachi, 1981).

Culture Environment

The ability to maintain zygote and embryo viability is an integral component of an *in vitro* fertilization system and is dictated by culture medium components and the external environment in which culture is allowed. Culture requirements appear to be highly species-specific (Gwatkin, 1972; Roger, 1978; Wright and Bondioli, 1981) with even slight modifications of medium markedly altering IVF rates.

The successful culture of oocytes and embryos is dependent on the presence and correct concentrations of ions (in salt form), proteins and energy sources (Biggers et al.,

1971; Brackett, 1981b). Ions necessary for successful IVF and/or embryo culture include: sodium, potassium, calcium, magnesium, chloride, phosphate and bicarbonate. Media generally are supplemented with serum or albumin as the protein source, a component probably also necessary for capacitating spermatozoa. Energy substrates for mouse embryos usually include pyruvate, lactate, glucose or other carbon chains, although there appears to be a difference in the utilization of these sources depending on stage of development (Brinster, 1973). Oocytes primarily metabolize pyruvate. Glycolysis in two-cell embryos is expanded to include pyruvate, lactate or oxaloacetate. At the eight-cell stage, embryos develop the capacity to utilize glucose or other sugars while blastocysts (like most somatic cells) can undergo glycolysis using carbon chains, fatty acids or amino acids.

Culturing in a relatively anaerobic, humidified environment (5% CO_2 in air) at 37 or 38°C appears to be the most successful and routinely used incubation condition (Biggers et al., 1971; Gwatkin, 1972; Brackett, 1981b; Wright and Bondioli, 1981). The microdrop method (Brinster, 1963) generally is the most commonly used for oocyte/embryo culture. In this procedure, the gametes/embryos are placed in 25 to 100 µl volumes of medium immersed in lightweight paraffin oil, thereby maintaining a constant temperature and pH while avoiding medium evaporation.

Synchronization of the Recipient

Closely synchronized estrous cycles (± 24 hours) between the embryo donor and recipient are essential to successful embryo transfer as documented in a variety of species including the pig (Webel et al., 1970), rabbit (Adams, 1971), cow (Newcomb and Rowson, 1976), mare (Voss et al., 1979) and mouse (Whittingham, 1979). Asynchronous transfer (greater than 24 hours) usually results in early embryonic mortality as a result of an improper oviductal or uterine environment.

IN VITRO FERTILIZATION OF CARNIVORE OOCYTES

Within the order Carnivora, IVF has been accomplished only in the dog and cat. The birth of offspring resulting from the transfer of *in vitro* fertilized carnivore ova has not been reported.

Canine

The *in vitro* fertilization of dog ovarian oocytes matured *in vitro* has been reported by Mahi and Yanagimachi (1976, 1978). Culturing dog oocytes for 0 to 72 hours in a TC-199 medium supplemented with 20% fetal calf serum increased the incidence of GVBD over time (Mahi and Yanagimachi, 1976). After transfer to a Biggers', Whitten's and Whittingham's (BWW) medium, the rate of sperm penetration or swelling of the sperm head within the vitellus was not influenced by the meiotic status of the oocyte. However, modification of the original salt, buffer, glucose and bovine serum albumin components of the BWW medium to give a defined canine capacitation medium (CCM) greatly enhanced spermatozoal motility and the percent of acrosome-reacted spermatozoa after a 7 hour incubation (Mahi and Yanagimachi, 1978). Additionally, CCM significantly increased the proportion of zona-penetrated ova (CCM: 71.4%; BWW: 22.2%). No data were provided on the proportion of oocytes containing swollen sperm heads or the cleavage rate of the cultured oocytes.

Feline

Hamner and associates (1970) were the first to report the IVF of feline oocytes. Using an exogenous hormonal regimen consisting of 150 IU PMSG injected subcutaneously followed by 50 IU hCG (intramuscularly) 72 hours later, oocytes were flushed from the oviducts and placed in Brackett's medium (Brackett, 1970) under oil in a 5% CO_2 in air, humidified environment at 37°C. Ejaculated spermatozoa, capacitated in the

uterine horns of naturally estrous females for 0.5 to to 24 hours were retrieved by flushing and added to the oocytes at concentrations of 15 to 75 X 10^5 spermatozoa/ml. After an 18 hour incubation, oocytes were removed and examined for cleavage. Freshly ejaculated, uncapacitated spermatozoa were incapable of fertilization *in vitro*. Sperm cells, previously incubated *in utero* for 0.5 hours, fertilized oocytes in one of three trials. Uterine incubation of spermatozoa for 2 to 24 hours resulted in cleavage rates ranging from 53 to 90%. *In vitro* fertilized embryos developed to the 16-cell stage but degenerated after 3 days in culture.

Bowen (1977) compared the rate of IVF using ductus deferens spermatozoa suspended in two different media (a modified Biggers', Stern and Whittingham, mBSW; and a modified Ham's F-10). After treating anestrous females with PMSG (100 or 150 IU intramuscularly) followed 72 hours later by commercially available, exogenous LH (2.5 mg intramuscularly), oocytes were flushed from the oviducts and cultured in one of the two spermatozoal suspensions containing 5.5 to 23 X 10^5 sperm/ml. After a 12 to 14 hour culture interval at 38°C, ova were removed from the spermatozoa and assessed for fertilization at 6 hour intervals. The presence of two or more blastomeres was indicative of successful fertilization. Each medium supported an equivalently high rate of fertilization (mBSW, 77.8%; mHam's F-10, 80.1%). Although percent culture rates were not available, embryos allowed further cleavage developed to the blastocyst stage while none of 25 control (non-inseminated) oocytes underwent parthenogenetic cleavage.

Niwa et al. (1985) recently reported observations on the early events of *in vitro* fertilization of ovulated feline oocytes mixed with epididymal spermatozoa. Following a hormonal regimen of PMSG (150 IU intramuscularly) and hCG (100 IU intramuscularly) 72 hours later, oocytes were collected from oviductal flushes 31 to 32 hours post-hCG. Diluted spermatozoa (0.2 to 1.8 X 10^6/ml), collected from the caudae epididymides of castrated males, were placed with the oocytes in a modified Kreb's Ringer bicarbonate (mKRB) medium in a 37°C, 5% CO_2 in air, humidified environment and cultured at

intervals ranging from 0.25 to 5 hours. Following incubation, cumulus cells were removed with hyaluronidase (0.1%) treatment, and the oocytes were fixed in 25% acetic alcohol, stained with aceto-orecin (1%) and examined for sperm penetration. Oocyte penetration occurred as early as 30 minutes following insemination indicating that the sperm capacitation time within this culture environment and before zona pellucida penetration was relatively short (15 to 20 minutes).

In vitro fertilization has not been reported for cat oocytes retrieved directly from ovarian follicles. If laparoscopic recovery of feline ovarian oocytes could be accomplished as routinely performed in women (Wood et al., 1981; Mettler et al., 1982; Lauritsen, 1983), then unnecessary trauma to the reproductive tract could be avoided. Repeated surgeries, including oviductal and/or uterine horn flushes for oocyte recovery can cause severe tissue adhesions, compromising the future reproductive potential of the animal. This consideration is especially important if these oocyte/embryo technologies are ever to be applied to rare or endangered felids.

OBJECTIVES

The objective of this study was to integratively study gamete interaction and early embryonic development and the effects of exogenous gonadotropins on these physiological processes in a relatively unstudied carnivore, the domestic cat. Because this species serves as an animal model for developing biological concepts and methodologies potentially useful in facilitating the study and propagation of rare Felidae, the IVF system developed in the domestic cat also was tested in a genetically related, non-domestic felid, the leopard cat (*Felis bengalensis*). The latter species, similar in size and weight to the domestic cat, is indigenous to east Asia and the Philippine Islands (Walker, 1964).

This report consisted of two primary studies, the first concerned with embryo recovery and transfer and the second with *in vitro* fertilization. The former was designed to compare ovarian responsiveness, uterine morphology and embryo recovery and quality between cats experiencing a natural estrus (NE) and those induced into estrus (IE) with a commercial source of exogenous follicle stimulating hormone (FSH-P). In addition, to determine the ability of gonadotropin-treated recipients to establish and maintain pregnancy, embryos from NE and IE donors were transferred to conspecific surrogates. To evaluate the quantitative and qualitative alterations of circulating steroids, serum concentrations of estradiol -17ß and progesterone from cats in natural estrus were compared to those experiencing a gonadotropin-induced estrus.

The second study was designed to evaluate the efficacy of laparoscopically collecting mature feline ovarian oocytes from gonadotropin-treated females and to determine the ability of these gametes to become fertilized *in vitro*. To assess the influence of exogenous hormones on the oocyte's ability to fuse with freshly ejaculated homologous spermatozoa, IVF rates were compared among cats given PMSG and hCG at two different intervals and two hCG doses. To determine if functional CL were formed following follicular aspiration, the developmental morphology and progesterone secretion of luteal

tissue from PMSG-treated cats were compared to females in a natural estrus. To test the developmental competence of cat zygotes fertilized *in vitro* and the ability of gonadotropin-treated females subjected to follicular aspiration to establish and maintain a normal maternal-fetal relationship, embryos produced from IVF were transferred to original oocyte donors and the females monitored for pregnancy. Finally, to determine the feasibility of adapting the domestic cat system to a non-domestic felid, IVF was attempted in the taxonomically-related leopard cat. This cat's non-endangered status (CITES, 1973), similar body size and ability to hybridize with the domestic cat (Benveniste and Todaro, 1975) suggests that this species is a logical first choice for adapting IVF technology to a nondomestic Felidae species.

MATERIALS AND METHODS

Animal housing conditions and experimental methods were approved by the Veterinary Resources Branch-Animal Welfare Committee of the National Institutes of Health.

ANIMAL HOUSING AND MAINTENANCE

Adult domestic cats were housed individually (males) or in groups of two to four (females) in stainless-steel cages (83 cm high X 78 cm wide X 91 cm deep) containing a metal shelf (78 cm wide X 21 cm deep). All individuals had access to a dry, commercial cat food (Purina Cat Chow, Ralston Purina Co., St. Louis, MO) and water *ad libitum*. Because the colony rooms contained windows, cats were exposed to natural fluctuations in photoperiod; additionally, the room was supplemented with 12 hours of artificial fluorescent illumination daily.

LAPAROSCOPY

Before any specific hormonal treatment, all female cats were subjected to a laparoscopic examination to evaluate ovarian activity using equipment purchased from the Richard Wolf Medical Instrument Company (Rosemont, IL). Details for a combined ketamine hydrochloride (ketamine HCl; Vetalar®, Parke-Davis, Morris Plains, NJ)/acepromazine maleate (Ayerst Labs, Rouses Pt., NY) anesthesia (10:1 ratio, 15 to 20 mg/kg and 1.5 to 2 mg/kg body wt.) have been published (Wildt et al., 1977; Wildt and Seager, 1980). Supplemental doses to maintain general anesthesia ranged from 0.4 to 0.5 mg/kg ketamine HCl and 0.04 to 0.05 mg/kg of acepromazine. Using sterile technique, a pneumoperitoneum was established with 100% CO_2 via a Verres needle approach. A 180° laparoscopic telescope (5 mm in diameter) then was inserted into the abdominal cavity

through a 1.5 cm incision made in the umbilical area via a trocar-cannula unit. The ancillary Verres needle probe (2 mm in diameter), inserted adjacent to the laparoscope was used to manipulate the reproductive organs into view and also aided in estimating ovarian follicle size. At the end of the examination, all instruments were removed from the abdominal cavity, the incision site was closed with absorbable suture material and 1 ml (100,000 units) of antibiotic (Flocillin, Bristol Veterinary Products, Syracuse, NY) injected subcutaneously. Animals were monitored closely during the recovery period and supportive therapy administered if necessary.

STUDY 1. FELINE EMBRYO COLLECTION AND TRANSFER

Ovulation induction, embryo collection and transfer

Adult female domestic cats were assigned to one of two groups: 1) induced estrus (IE, n=30) or 2) natural estrus (NE, n=29). After a pre-treatment laparoscopy (Day -6) queens in the IE group with inactive ovaries (no follicular or luteal activity) or with follicles less than 2 mm in diameter received 2 mg FSH-P (Burns-Biotec, Lincoln, NE) intramuscularly once daily for 5 days (Days -5 to -1) to induce ovarian activity and estrous behavior (Wildt et al., 1978b). Beginning on the first day of estrus (Day 1), embryo donors were allowed single copulations with intact adult males three times/day at 3 to 4 hour intervals for 3 days. To induce ovulation in synchrony with the donors, females designated to subsequently receive embryos (recipients, n=15) were injected with 250 IU hCG intramuscularly (Sigma Chemical Co., St. Louis, MO) at the time of the second copulation on the second and third day of estrus.

Donor and recipient females were subjected to a second laparoscopic examination on Day 4 or 5 to confirm ovulation and count the number of CL and unovulated follicles (UF, >2 mm in diameter) present on the ovaries. Uterine tone also was assessed on a subjective scale of 1 to 4: 1=turgid, enlarged with distinct segmental swellings; 2=turgid

and enlarged with segmental swellings less prominent; 3=slight turgidity and no segmental swellings; 4=flaccid, small diameter and smooth.

To collect feline embryos from uterine flushes, the reproductive tracts of donor females were exposed by laparotomy on Day 7 or 8. Females were induced into a surgical plane of anesthesia using the previously described ketamine HCl/acepromazine mixture. After intubation with a 3.5 mm in diameter endotracheal tube, a surgical plane of anesthesia was maintained with 1 to 2% halothane (Halocarbon Laboratories Inc., Hackensack, NJ) mixed with 0.8 to 1% oxygen. After the reproductive organs were exposed, a flanged tomcat catheter (Sovereign, 3.5 French, Monoject, Division of Sherwood Medical, St. Louis, MO) was inserted into the caudal terminus of the uterine horn (approximately 2 to 3 cm cranial to the uterine bifurcation) through the myo- and endometrium into the uterine lumen. After placement of a 17 gauge catheter into the uterine lumen near the utero-tubal junction, 25 to 30 ml of warmed (37°C) Ham's F-10 medium (NIH Media Unit, Bethesda, MD) containing 5% heat inactivated, filtered fetal calf serum (Gibco Laboratories, Chagrin Falls, OH) were flushed in a normograde direction through the uterine horns and collected into 12 ml (60 X 15 mm) gridded plastic tissue culture dishes (Corning Glassworks, Corning, NY). Using a Wild M5APO stereomicroscope (Bunton Instrument Company, Rockville, MD), the dishes were methodically searched (12X) and all embryos or unfertilized ova (UFO) transferred into fresh medium using a hand-held glass pipette (from 3 mm inner diameter glass, Corning Glassworks, Corning, NY) controlled by gentle mouth suction. Each embryo was assessed for developmental stage and assigned a quality grade based on morphological appearance: 1=excellent, 2=good, 3=fair, 4=poor or degenerate. The following criteria, based on earlier cattle embryo studies (Elsden et al., 1978; Linder and Wright, 1983), were used to assign quality grades:

Grade 1: embryo at the expected stage of development, symmetrical with blastomeres of uniform size and shape.

Grade 2: embryo at the expected stage of development or slightly retarded with some imperfections such as a few extruded or irregularly shaped blastomeres or vesicles.

Grade 3: embryo 1 to 2 days behind the expected stage of development, irregular in shape, blastomeres of non-uniform size and shape, numerous extrusions.

Grade 4: embryo 2 or more days behind the expected stage of development, numerous extrusions and/or degenerate cells with blastomeres irregular in color and vesiculated.

After embryo quality grading, recipient females were injected with the ketamine/acepromazine mixture, and a surgical plane of anesthesia maintained with halothane and oxygen (as described for the donor females). For delivery into the uterine horns of the recipient, the embryos were aspirated into a tomcat catheter attached to a 1 ml plastic syringe. To avoid accidental embryo loss, the catheter was loaded with a bilateral buffer of air (2 mm) and medium (10 µl) surrounding the embryos. After exposing the reproductive tract at laparotomy, each uterine horn of the recipient was punctured with a 17 gauge needle within a catheter (approximately 1 to 2 cm caudal to the utero-tubal junction). The tomcat catheter was inserted into the uterine horn via this puncture and the embryos expelled into the uterine lumen by depressing the syringe plunger. The laparotomy site was sutured and antibiotic given subcutaneously (1 ml Flocillin). Pregnancy was diagnosed 35 to 40 days later by abdominal palpation, and pregnant queens were allowed to carry kittens to term.

Females in the NE group were monitored daily for signs of estrous behavior (i.e., increased vocalization, rubbing, lordosis and treading of the hind limbs when exposed to a male; Michael, 1961; Wildt et al., 1978a., Shille et al., 1979). At the onset of estrus, donor females were subjected to the same mating, ovulation induction (mating or hCG), embryo collection and transfer procedures as described for the IE females.

Endocrine profiles

To determine endocrine patterns in cats experiencing a natural versus induced-estrus, blood samples were collected from IE (n=8) and NE (n=9) female cats. Blood (3 ml) was collected twice daily from IE donor females on Day -6 through Day 6 (at approximately 0830 and 1630 hours) by jugular venipuncture and placed into serum separation tubes (Venoject, Terumo Medical Corp., Elkton, MD). The same bleeding schedule was followed for the NE females but from the first day of estrus through Day 6. After centrifugation (1200 X g), the sera were removed and stored in individually labelled vials (Bio-Vial, Beckman Instrument Co., Palo Alto, CA) at -20°C until assayed. Sera were analyzed for estradiol-17ß and progesterone concentrations using commercially available radioimmunoassay kits (Radioassay Systems Laboratories, Inc., Carson, CA).

The estradiol-17ß assay kit detected only the unconjugated form of this steriod, but did not distinguish between free and protein-bound estradiol. The reagents in the assay included: 1) a phosphosaline gelatin diluent buffer, pH 6.1, containing rabbit gamma globulin and 0.1% sodium azide as a preservative; 2) estradiol-17ß antibody, generated by injecting 6-keto-estradiol-17ß-6-oxime bovine serum albumin (BSA) into rabbits to obtain antiserum; 3) seven estradiol-17ß standards: 0 pg/ml; 10 pg/ml; 30 pg/ml; 100 pg/ml; 300 pg/ml; 1000 pg/ml and 3000 pg/ml in a BSA buffer solution; 4) a precipitating solution (second antibody consisting of goat-anti-rabbit gamma globulin and polyethylene glycol in a tris-based buffer); and 5) ^{125}I labelled estradiol-17ß. The cross-reactivities for the estradiol antibody were: estradiol-17ß, 100%; estrone, 20.0%; estriol, 1.51%; estradiol-17α, 0.68%; ethinyl estradiol, testosterone, 5α-dihydrotestosterone, cholesterol, pregnenolone, 17-hydroxypregnenolone, progesterone, 17-α-hydroxyprogesterone, 20-α-dihydroprogesterone, 11-desoxycortisol, cortisol, aldosterone, androstendione, dihydroepiandrosterone and dihydroepiandrosterone sulfate, <0.01%. Individual assays were carried out in 96 consecutively numbered 12 X 75 mm borosilicate glass tubes (Kimble, Division of Owens-Illinois, Toledo, OH).

After bringing all reagents to room temperature, 500 µl of diluent buffer were added to tubes #1 and 2 and 50 µl of the 0 pg/ml estradiol-17ß standard were added to tubes #1 to 4. The estradiol standards were added to tubes #5 to 16 (50 µl of each standard to two tubes) and 50 µl of each sample were aliquoted individually into the remaining tubes. After 500 µl of the radioactively-labelled estradiol-17ß were pipetted into each tube, 500 µl of the anti-estradiol-17ß were pipetted into all tubes, which were vortexed for 5 seconds. After a 90 minute incubation in a 37°C water bath, 500 µl of the precipitant solution were added to all assay tubes which were vortexed and centrifuged at 2500 rpm (1000 X g) for 20 minutes. After decanting the supernatant, all tubes were inverted and drained on absorbent paper for 10 minutes, wiped free of excess liquid and counted for 1 minute in an LKB gamma counter (LKB 1271 RIAGAMMA , Division of Wallac, Wallac Oy, Turku, Finland) to quantify radioactivity. The counter automatically calculated a standard curve from the standard solution counts and, based on these values, calculated the amount of estradiol-17ß in each unknown sample. The inter- and intra-assay coefficients of variation for this assay were 1.7% (n=5) and 3.2% (n=6), respectively.

The reagents used in the progesterone assay were: 1) a diluent buffer consisting of 0.005% rabbit gamma globulin in 0.1 M phosphosaline-gelatin buffer; 2) progesterone antibody generated by injecting 11-α-hydroxyprogesterone-11-α-hemisuccinate-human serum albumin into rabbits; 3) seven progesterone standards: 0.0 ng/ml; 0.2 ng/ml; 0.5 ng/ml; 2.0 ng/ml; 5.0 ng/ml; 10.0 ng/ml and 40.0 ng/ml; 4) a precipitating antiserum (second antibody) made from goat anti-rabbit gamma globulin in 0.01 M phosphosaline buffer (pH 7.5); and 5) ^{125}I labelled progesterone. The reported cross-reactivities of the first antibody were: progesterone, 100%; 20-α-dihydroprogesterone, 6.25%; desoxycorticosterone, 3.20%; corticosterone, 0.42%; 17-α-hydroxyprogesterone, 0.15%; pregnenolone, 0.06%; androstendione, 0.04%; testosterone, 0.03%; 11-desoxycortisone, pregnenolone sulfate, cholesterol, dihydroepiandrosterone, etiocholanolone, estradiol-17ß,

estradiol-17α, estrone, estriol, androsterone, aldosterone and cortisol, <0.01%. The assay was carried out in 96 consecutively numbered 12 X 75 mm borosilicate glass tubes.

After all reagents were brought to room temperature, 100 µl of the 0 ng/ml standard were added to tubes #1 to 4. Progesterone standards were added to tubes #5 to 16 (100 µl aliquots of each standard into two tubes). After pipetting 100 µl of the serum samples individually into tubes #17 to 96, 500 µl of assay buffer were placed in tubes #1 and 2 and antiprogesterone (500 µl) to tubes #3 to 96. After shaking the test tube racks for 30 seconds, 200 µl of the ^{125}I labelled progesterone were pipetted into all assay tubes which then were vortexed and incubated in a 37°C water bath for 60 minutes. Second antibody (100 µl) then was placed into all tubes which were vortexed and allowed a second (60 minute) incubation. All tubes were centrifuged, counted and unknowns calculated as described above for the estradiol-17ß assay. Inter- and intra-assay coefficients of variation for this assay were 12.4% (n=4) and 7.0% (n=8), respectively.

Data analysis

The mean number of CL and anovulatory follicles/female and the mean number of embryos plus UFO/female producing embryos between the NE and IE groups were compared using a Student's t test (Snedecor and Cochran, 1980). The uterine tone values, percent embryo recovery rate (number of embryos + UFO/number of CL) and proportion of transferrable (QG 1 to 3) and poor (QG 4) quality embryos collected were evaluated by Chi Square analysis (Snedecor and Cochran, 1980). Estradiol-17ß profiles on Day 1 of estrus were compared using a Student's t test. Progesterone profiles were analyzed using a split-plot design with a statistical analysis computer program (SAS, 1982).

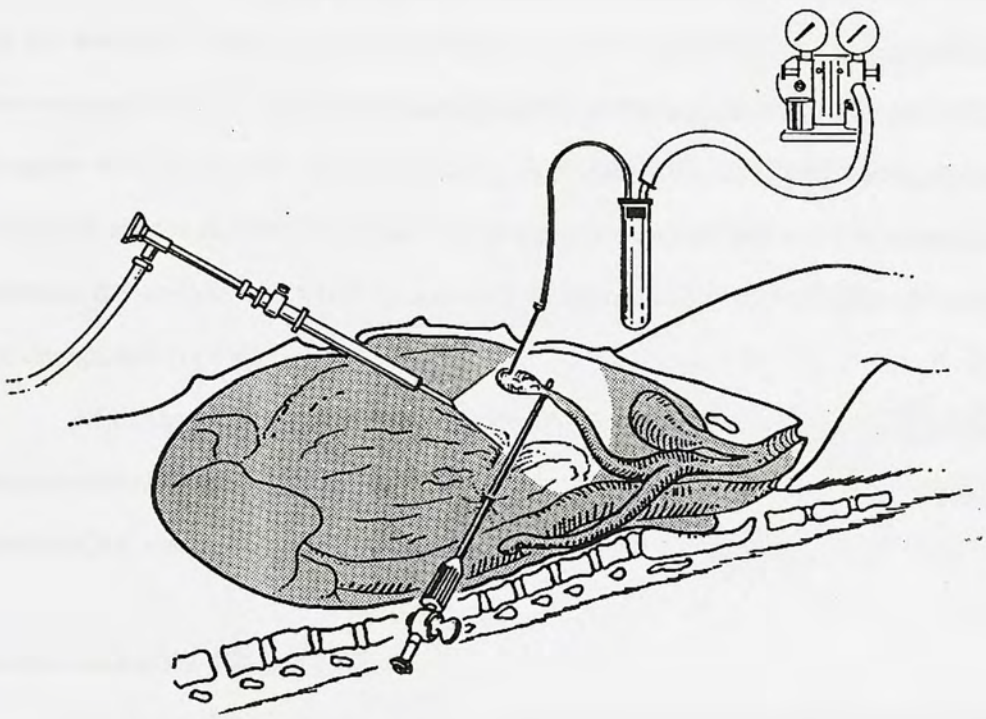
STUDY 2. *IN VITRO* FERTILIZATION OF FELINE OOCYTES

Induction of ovarian activity and follicular aspiration

Before exogenous gonadotropin treatment, ovarian activity was assessed in adult cats by laparoscopy (as described above) (Day 0). Only females with completely inactive ovaries (no follicles or luteal tissue present) or ovaries containing follicles less than 2 mm in diameter were selected for gonadotropin treatment (n=55). A single intramuscular injection of PMSG (150 IU, Sigma Chemical Co., St. Louis, MO) was administered on Day 1 followed 72 or 80 hours later by either 100 or 200 IU of hCG (intramuscularly) to reinitiate meiosis. Each cat received PMSG followed by one of four hCG treatments: 1) 100 IU hCG 72 hours later; 2) 200 IU hCG 72 hours later; 3) 100 IU hCG 80 hours later; 4) 200 IU hCG 80 hours later. Beginning 24 hours after PMSG administration, each queen was checked twice daily for behavioral indications of estrus.

On Day 5 and precisely at 25 to 27 hours after hCG, each animal was subjected to laparoscopy to permit oocyte collection by follicular aspiration. General anesthesia was induced and maintained with the ketamine HCl/acepromazine maleate mixture described for Study 1. Follicular aspirations were accomplished using a 22 gauge needle attached to size 100 polyethylene tubing (inner diameter, 0.86 mm; Clay Adams, Division of Becton-Dickinson, Parsippany, NJ), a siliconized collection tube (Terumo Medical Corporation, Elkton, MD) and a vacuum pump (GAST Manufacturing Corp., Benton Harbor, MI; Fig. 2). Before aspiration, the needle and tubing were rinsed with 2 to 3 ml of warmed (37°C) modified Kreb's Ringer bicarbonate medium (mKRB; Niwa et al., 1985; Appendix I) containing 40 units heparin/ml of medium. While stabilizing the reproductive tract with the accessory Verres needle probe, the aspiration needle was inserted through the abdominal wall at a site ventro-medial to the ovary. Ovarian follicles greater than 2 mm in diameter with distinct borders and moderate to pronounced vascularization on the follicular wall were punctured by perforating the follicular apex with the needle, bevel side down, while

Figure 2. Laparoscopic aspiration of domestic cat ovarian follicles.



applying gentle negative pressure (100 mm Hg) via the vacuum pump assembly. The aspiration needle was rotated gently within each follicle to allow complete suction of the contents. The needle was removed and, with the tubing apparatus, internally rinsed with 3 to 4 ml of medium. The collection tube was placed into a 37°C incubator and, after replacing the collection tube and aspiration needle, the procedure was repeated for the contralateral ovary. Each collection tube was emptied into a gridded, plastic Petri dish (as described for embryo searching) and rinsed with 3 ml of medium. The dishes were immediately searched by stereomicroscopy and identified oocytes were transferred to fresh mKRB medium (containing no heparin) and placed in a 5% CO_2 in air, humidified environment at 37°C. To assess maturity, each oocyte and its surrounding cumulus cell complex were examined morphologically. A mature oocyte was considered as one in which the corona radiata and cumulus oophorus cells were loosened and expanded; in contrast, any oocyte with a tightly compacted corona radiata was considered immature and not designated for IVF.

After classification, mature oocytes were washed three time in mKRB under oil to remove contaminants including red blood cells and debris. After placing the oocytes in fresh mKRB medium, the culture dish was returned to the incubator.

Semen collection and preparation

On each collection day and after mature oocytes were identified, an adult male cat was anesthetized and subjected to electroejaculation as described previously (Platz et al., 1978; Wildt et al., 1983; Carter et al., 1984). After inducing a surgical plane of anesthesia with ketamine HCl (20 to 25 mg/kg), a lubricated Teflon® probe (3 cm diameter X 13 cm length, P.T. Electronics, Boring, OR) with three 2 mm wide longitudinal electrodes was inserted into the rectum with the electrodes oriented ventrally. Using an AC, 60 Hz sine-wave ejaculator, a regimented electroejaculation sequence consisting of a total of 80 electrical stimuli was applied in three series (Wildt et al., 1983). Two series of 30 stimuli

and one of 20 stimuli were applied in sets of 10 stimulations at increasing increments of voltage and amperage. Stimuli were given in a 3 second on and 3 second off pattern with a continuous rise in voltage from zero to the desired peak and returning to zero volts. Voltages of two, three and four were used in the first series, increased to three four and five in the second series and maintained at four and five volts for the last series. A 2 to 3 minute rest period was allowed between series.

Ejaculates from each series were collected in separate, sterile 5 ml plastic vials (Nalge Co., Division of Sybron Corp., Rochester, NY) and combined immediately after the third electroejaculation series. A 10 µl aliquot was placed on a microscope slide under a cover slip and examined (25X) to subjectively estimate spermatozoal percent motility and progressive status on a scale of 5 to 0 as follows: 5=rapid, linear forward progression; 4=steady, forward progression; 3=side-to side movement with forward progression; 2=moderate side-to-side movement with slow forward progression; 1=slight side-to-side movement with no forward progression; 0=no forward progression or movement. Only sperm samples with at least a 70% motility and 3.5 progressive status rating were designated for further IVF processing. After transfer into a 1.5 ml plastic, conical tube (Sarstedt Inc., Princeton, NJ), the ejaculate was subjected to a "swim-up" procedure (Lopata et al., 1974; Makler et at., 1984) which involved dilution with an equivalent volume of mKRB (equilibrated to 23°C) and centrifugation for 8 minutes at 300 X g. After aspirating and discarding the supernatant, 150 µl of mKRB was slowly and carefully layered onto the resulting pellet and the sperm allowed a 1 hour swim-up at room (21 to 23°C) temperature. The layered medium component was gently aspirated from the pellet surface and evaluated for spermatozoal percent motility and progressive status as well as sperm concentration/ml as calculated by the hemacytometer method (Wildt et al., 1983). Based on cell count, the spermatozoal solution was diluted to a final concentration of 2 X 10^5 sperm cells/ml.

Fertilization

Fertilization involved transferring a 100 µl drop of the mKRB-diluted sperm suspension (2 X 10^4 sperm cells/drop) into a Falcon 3001, 35 X 10 mm Petri dish (Falcon, Division of Becton-Dickinson, Oxnard, CA) under lightweight paraffin oil (Fisher Scientific Co., Fairlawn, NJ), previously saturated with 5% CO_2 by bubbling the oil for 15 minutes with this gas. Oocytes from the four treatments (a total of 101, 101, 106 and 108, respectively) were maintained in separate sperm drops (10 oocytes or fewer/drop). Control ova (n=32, taken from all treatments for determining the rate of parthenogenetic cleavage) were placed under oil into a drop of mKRB containing no spermatozoa. All dishes were placed into an incubator environment of 5% CO_2 in humidified air and maintained at 37°C. Eighteen to 20 hours later, the oocytes were removed from the fertilization dishes and washed three times in a 0.2% hyaluronidase (Type 1-S, from bovine testes; Sigma Chemical Corp., St. Louis, MO) solution in mKRB medium to remove residual cumulus cells (control oocytes were washed first to avoid introducing spermatozoa). Specifically, the washing procedure entailed transferring the oocytes into the enzyme solution for 1 to 3 minutes and applying gentle back and forth suction with a 200 micron diameter, mouth-controlled pipette to remove cumulus cells and loosely attached spermatozoa. Oocytes were returned to the incubator in 100 µl drops of fresh mKRB under oil and re-examined 24 to 30 hours post-insemination for cleavage. At this time, oocytes appearing fragmented or distinctly irregular in shape were classified as degenerate.

Oocytes failing to cleave were treated with a Hoescht stain specific for DNA (Pursel et al., 1985). This procedure involved counterstaining the oocytes for 1 to 2 minutes with a 0.1% Trypan blue solution dissolved in 2.3% sodium citrate (Sigma Chemical Corp., St. Louis, MO) to quench the fluorescence of the attached spermatozoa and cumulus cells. The oocytes then were exposed for 15 minutes to the Hoescht stain (100 µl of 2.3% sodium citrate to 10 µl of Hoescht stock: 1 mg Hoescht powder/ml water; #33342, Sigma Chemical

Corp., St. Louis, MO) which fluoresced DNA as a bright blue color at an ultraviolet excitation of 340 to 380 λ.

Following a 3 minute centrifugation (15,000 X g) in a fixed-speed microfuge (Eppendorf Microcentrifuge, Brinkmann Instrument Co., Westbury, NY) to displace cellular lipid, the oocytes were examined using differential interference contrast (DIC) and fluorescence optics (25 and 40X, Leitz Laborlux 12, Bunton Instrument Co., Rockville, MD) to identify germinal vesicles, polar bodies or pronuclei. Because cat oocytes contain a high proportion of dark, dense lipid (Guraya, 1965), recognizing intracellular germinal vesicles or pronuclei is impossible without cytoplasm displacement. Wall et al. (1985) first reported that centrifuging pig oocytes, which also are lipid dense, allows identification of pronuclei without interfering with the developmental capacity of the embryo. Because the Hoescht stain is specific for DNA, only structures containing chromatin (i.e. germinal vesicles, pronuclei, polar bodies, spermatozoa or granulosa cells) were visible with fluorescence optics. Based on structure location and intensity of fluorescence, observations made with DIC optics after ova centrifugation indicating the presence of polar bodies or pronuclei were validated with the Hoescht stain. Criteria for fertilization included the presence of two polar bodies, two pronuclei or cleavage to at least the two-cell stage.

To test the developmental competence of *in vitro* fertilized cat embryos, a portion of the cleaved embryos were selected for embryo culture or embryo transfer. Two-cell embryos designated for culture were placed in 100 µl of mKRB medium under oil and allowed to develop in a 5% CO_2 in air, humidified environment at 37°C for 1 to 3 days. Because feline embryos are homogeneously dark in appearance, counting individual blastomeres was difficult beyond the four-cell stage. After removal from culture, embryos were subjected to the Hoescht stain, flattened under a cover slip and examined by fluorescence microscopy for the presence of nuclei. Four sets of two- to four-cell embryos containing 18, 13, eight and seven embryos/set were designated for autotransfer to four original oocyte donors (30 to 42 hours after insemination). The donors were subjected to

general anesthesia as previously described and the oviducts exposed at laparotomy. The embryos (in 2 µl of phosphate buffered saline containing 3 mg/ml bovine serum albumin) were aspirated into size 50 PE tubing and the latter inserted bilaterally into the fimbriated end of the oviducts (three to nine embryos per oviduct). The incision site was sutured and antibiotics and supportive therapy provided as described previously. Pregnant queens were allowed to carry kittens to term.

Assessment of corpus luteum formation and function after follicular aspiration

To determine if normal CL were formed in gonadotropin-treated queens after follicular aspiration, ovaries were monitored over time for luteal development and regression and serial blood samples were analyzed for progesterone concentration. For gonadal examination, each of 11 queens was subjected to general anesthesia and routine laparoscopy using earlier described techniques. On the day of oocyte aspiration, a record was made of the number and sites of each aspirated follicle on the ovarian surface. Each animal then was laparoscopically examined at 7 day intervals for 6 weeks (the approximate maximum duration of a sterile luteal phase in a naturally mating queen; Wildt et al., 1981a) and the number and location of each CL noted and photographed using a SLR 35 mm camera and laparoscopic adapter (Richard Wolf Medical Instruments Corp., Rosemont, Il). Blood samples (3 ml) were obtained by jugular venipuncture (between 0800 and 1030 hours) from the same females (six receiving 100 IU hCG, five receiving 200 IU hCG) on Days 0 (day of pre-treatment laparoscopy), 1 (day of PMSG injection), 2, 3, 4 (day of hCG injection), 5 (day of follicular aspiration), 6 and 8. After Day 8, blood samples were collected every 48 to 72 hours for 8 weeks . Blood was processed as described previously, the sera stored at -20°C and later assayed for estradiol-17ß and progesterone using radioimmunoassays described earlier. Four naturally estrous cats, mated three times/day with a vasectomized male for the first 3 days of estrus (to induce ovulation) served as controls (NO group). Using this mating regimen, follicular rupture occurs on the

fourth or fifth day of estrus (Wildt et al., 1981a); therefore, the expected timing of the natural ovulations was presumed coincident (± 24 hours) with the timing of follicular aspiration. The NO queens were subjected to the same sequence of laparoscopic evaluations and serial blood sampling/estradiol-17ß and progesterone analysis as described for the PMSG/hCG group.

In vitro fertilization of leopard cat oocytes

Adult leopard cats were housed individually in wire enclosures 212 cm high X 135 cm wide X 275 cm deep, fed a commercial feline diet (Nebraska Feline diet, Central Nebraska Packing, Inc., North Platte, NE) and provided water *ad libitum*. These animals were exposed to natural fluctuations in daylength (10 to 12 hours daily) and 10 hours of supplemental artificial illumination daily. Each enclosure was equipped with a squeeze cage device (90 cm high X 60 cm wide X 70 cm deep) to facilitate animal containment for anesthesia induction and post-anesthesia recovery. For female leopard cats, the same dosage of ketamine HCl/acepromazine described for domestic cats was used. For electroejaculation of males, anesthesia was facilitated by the combined used of ketamine HCl (15-17/kg) and xylazine (Rompun, Haver, Miles Laboratories, Inc., Shawnee, KS, 0.3 to 0.5 mg/kg).

Each female (n=5) was subjected to laparoscopy prior to gonadotropin treatment and the ovaries examined for evidence of follicular or luteal development. Females with no follicles or follicles less than or equal to 2 mm in diameter were injected intramuscularly with 150 IU PMSG followed 80 hours later by 100 IU of hCG. Each leopard cat received the PMSG/hCG treatment twice at approximately a 60 day interval. The timing and laparoscopic approach for oocyte collection, evaluation and processing as well as for semen collection, sperm swim-up, insemination and culture were exactly the same as described for the domestic cat. Eighteen to 20 hours after insemination, the oocytes were washed with mKRB medium containing 0.2% hyaluronidase and then re-examined 6 to 12 hours later

for morphological evidence of cleavage. Uncleaved oocytes were Hoescht-stained and examined for the presence of germinal vesicles, polar bodies and pronuclei.

Data analysis

Differences in the mean number of follicles aspirated and oocytes collected between the two PMSG-hCG time intervals and the two hCG doses were analyzed using a Student's t test (Snedecor and Cochran, 1980). Variations within the four time interval/hCG dose treatments were analyzed further with a statistical software package (SYSTAT, Wilkinson, 1987) and one-way analysis of variance. Differences in the proportions of females exhibiting behavioral estrus, the percent oocyte recovery, and the proportion of oocytes with germinal vesicles or exhibiting polyspermy, degeneration, fertilization or cleavage were evaluated by Chi Square analysis (Snedecor and Cochran, 1980).

Individual progesterone profiles in either PMSG/hCG-treated queens subjected to follicular aspiration or naturally estrual-hCG-treated animals were plotted with a graphics software package (Cricketgraph, Version 1.0, Cricket Software, Philadelphia, PA) and the area under each curve calculated using a planimeter (Kueffel and Esser Co., Germany), with a coefficient of variation of 3.7% (n=5). Mean area under the curve for each treatment group was calculated and differences analyzed using a statistical software package by SYSTAT, which employed a one-way analysis of variance and a Student Neuman Kuehl's multiple range test (Steel and Torrie, 1960).

RESULTS

STUDY 1. FELINE EMBRYO COLLECTION AND TRANSFER

Reproductive characteristics, embryo recovery and quality

Table 2 provides a summary of estrous behavior, ovarian activity and uterine horn morphology of NE versus IE donor cats. The proportion of females allowing coitus was not different between groups ($P>0.05$), however, NE queens produced fewer CL and unovulated follicles than their IE counterparts ($P<0.01$). During the post-ovulation to embryo collection interval (Days 4 to 8), uterine horn tone rating differed ($P<0.01$) between the NE and IE groups (Table 2) which also was reflected in gross uterine appearance. The early luteal phase cornua of the NE cats were consistently more turgid, containing distinct segmental swellings (Fig. 3a) which contrasted to the smooth, flaccid uterine horns (Fig. 3b) of the gonadotropin-treated females.

Embryo recovery rate and the mean number of embryos recovered (from females producing embryos) were not different ($P>0.05$) between groups (Table 3). However, the NE cats proportionately produced more fertilized oocytes than the IE group ($P<0.01$). The NE cats produced primarily morula- to blastocyst-stage embryos while embryos from IE females were more retarded in development (Table 3, $P<0.01$). Only one of 14 NE females produced a single unfertilized ovum, whereas UFO were collected from over half (nine of 16) the IE females ($P<0.01$). Almost 25% of oocytes collected from IE cats were unfertilized. The proportion of embryos judged to be of transferrable quality (QG 1 to 3, Fig. 4a) was greater in the NE than the IE group (Table 4; $P<0.01$) while the IE group produced a greater percentage of poor quality (QG 4, Fig. 4b) embryos ($P<0.01$). Nevertheless, embryos collected from both NE and IE donors and transferred into synchronized recipients resulted in pregnancies (Table 4) including the birth of three kittens

Table 2. Reproductive characteristics of natural and gonadotropin-induced estrus cats

Group	No. of cats	No. mating (%)	Mean no. corpora lutea[a]	Mean no. unovulated follicles[a,b]	Mean uterine tone[a,c]
Natural estrus	29	26 (90)	5.3±0.5[d]	3.7± 1.1[d]	1.8± 0.2[d]
Induced estrus	30	28 (93)	9.4±0.9[e]	13.6± 2.4[e]	3.1± 0.2[e]

[a]Day 4 or 5 (Day 1=first day of estrus).

[b]>2 mm diameter

[c]Uterine tone 1=turgid, segmentally swollen, hypertrophied to 4=flaccid, smooth, and small diameter.

[d,e]Values within columns with different superscripts differ ($P<0.01$).

Table 3. Embryo recovery and quality from natural versus gonadotropin-induced estrus domestic cats

Group	No. females producing embryos	Embryo recovery rate/female [a]		Total	Fertilized ova		No. females producing unfertilized ova (%)	No. unfertilized ova (%)
		%	mean		No. 2-16 cells (%)	No. morulae/ blastocysts (%)		
Natural estrus	14	59.7±8.9	3.2±0.4	42	10 (23.8)[b]	32 (76.2)[b]	1 (7.1)[b]	1 (2.3)[b]
Induced estrus	16	57.0±9.4	4.4±0.7	62	35 (56.5)[c]	27 (43.5)[c]	9 (56.2)[c]	20 (24.4)[c]

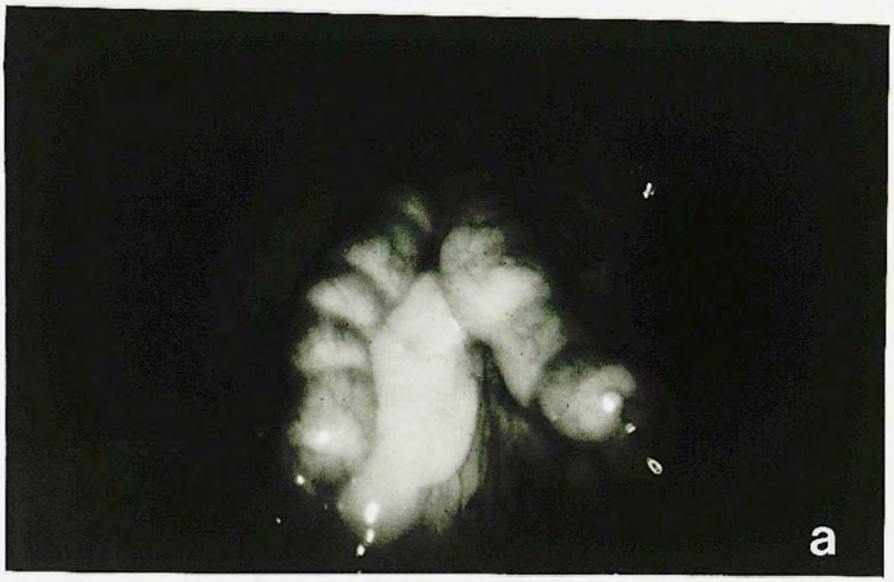
[a] % = No. embryos + no. unfertilized ova/no. CL; Mean = mean no.of embryos/cat which produced embryos.
[b,c] Values within columns with different superscripts differ (P<0.01).

Table 4. Embryo quality and transfer in natural and gonadotropin-induced estrus domestic cats

Group	Transferrable quality embryos[a] number (%)	Transferrable quality embryos[a] mean[b]	Poor or degenerate embryos[c] number (%)	Poor or degenerate embryos[c] mean[b]	No. pregnancies/ transfers (%)	No. embryos transferred/ term pregnancy	No. offspring
Natural estrus	33/42 (78.6)[d]	2.5± 0.5	9/42 (21.6)[d]	0.7± 0.4[d]	2/7 (29)	2	1
Induced estrus	30/62 (43.4)[e]	2.1± 0.5	32/62 (51.6)[e]	2.6± 0.6[e]	1/8 (13)	3	2

[a]Quality grades 1-3.
[b]Per cat which produced embryos.
[c]Quality grade 4.
[d,e]Values within columns with different superscripts differ (P<0.01).

Figure 3. Uterine cornuae 5 days after onset of a natural (a) or FSH-P induced (b) estrus domestic cats demonstrating uterine tone ratings of 1 and 4, respectively.


a

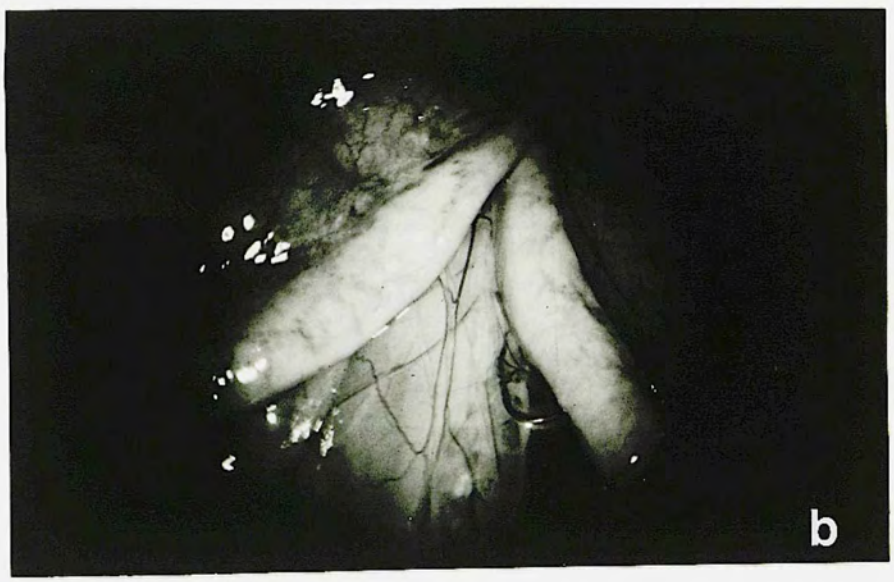
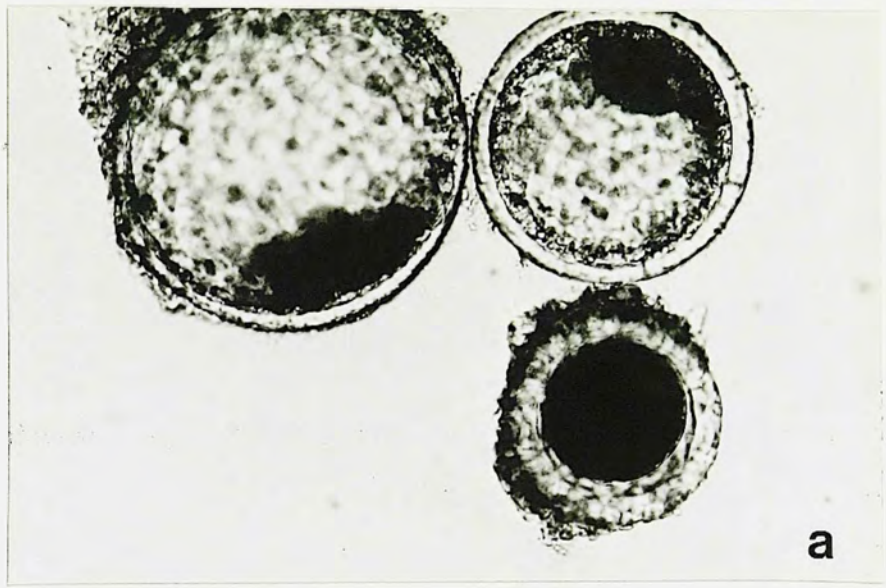

b

Figure 4. Domestic cat blastocysts and unfertilized ovum (a) and degenerate embryos (b) collected 8 days after the onset of mating.


a

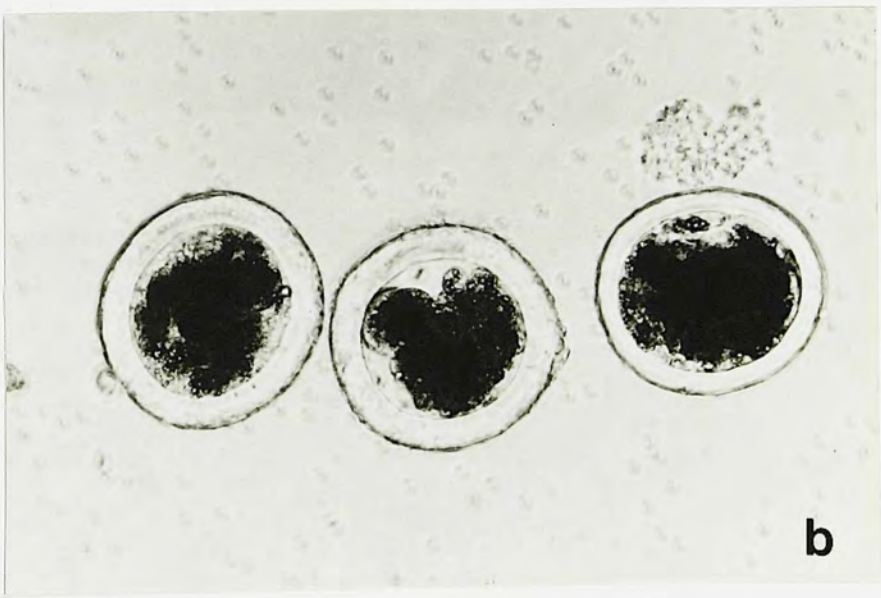

b

Figure 5. Kitten resulting from embryo transfer and surrogate mother.

from two queens after gestations of 66 and 68 days, respectively (Fig. 5). One recipient diagnosed pregnant by abdominal palpation apparently experienced fetal reabsorption.

Endocrine profiles

Figure 6 illustrates the mean serum estradiol-17ß and progesterone profiles from NE and IE cats. Although serum estradiol-17ß began to rise immediately after the onset of FSH-P treatment, maximal concentrations reached the levels observed in NE cats only on the afternoon of the last day of FSH-P treatment (Day -1). In the IE group, estradiol-17ß declined sharply after the cessation of gonadotropin treatment and by Day 1, mean estradiol-17ß concentration was more than two-fold greater in NE (72.9 ± 13.2 pg/ml) compared to IE queens (36.4 ± 5.9 pg/ml, Fig. 6, panel A). Serum progesterone remained at nadir concentrations during FSH-P administration but rose in the IE group coincident with the onset of estrus (Day 1). In contrast, the first detectable rise in serum progesterone for the NE controls occurred on the morning of Day 4 (Fig. 6, panel B), 72 hours after the premature elevation in the IE group.

STUDY 2. *IN VITRO* FERTILIZATION OF FELINE OOCYTES

In vitro fertilization of domestic cat follicular oocytes

Because of the extreme ovarian hyperstimulation and poor fertilization rates observed in FSH-P-treated cats in Study 1, PMSG was selected for ovarian stimulation in this study. Overall and without regard to specific treatment, slightly more than half (52.7%) the gonadotropin-injected females exhibited behavioral estrus, and an average of 11.7 ± 0.7 mature follicles (Fig. 7) were observed 25 to 27 hours after hCG. A high proportion (91.4%) of the oocytes from these follicles were recovered (overall mean, 10.7 ± 0.7 oocytes/cat). When analyzed on the basis of gonadotropin treatment, neither the time interval between PMSG and hCG nor the hCG dose influenced ($P>0.05$) the proportion of

Figure 6. Mean (± S.E.M.) serum estradiol-17ß (a) and progesterone (b) profiles from domestic cats in natural (●) or FSH-P induced (o) estrus.

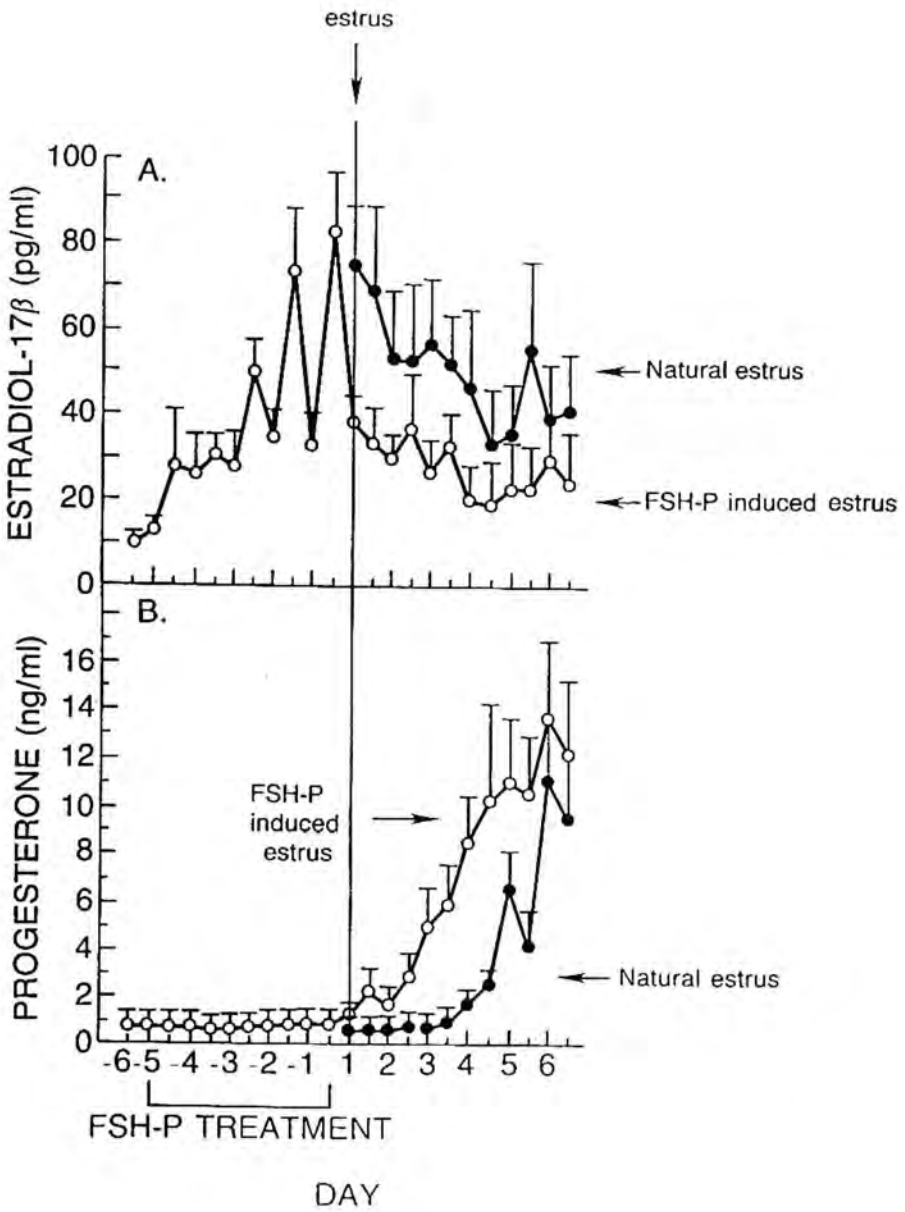
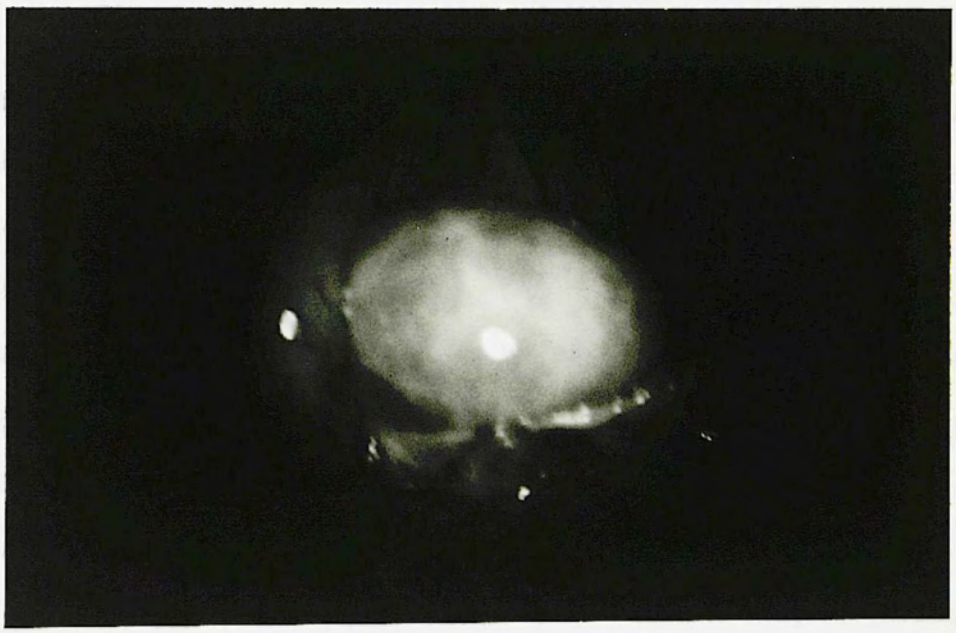

estrus
A.
ESTRADIOL-17β (pg/ml)
100
80
60
40
20
0
Natural estrus
FSH-P induced estrus
B.
PROGESTERONE (ng/ml)
16
14
12
10
8
6
4
2
0
FSH-P
induced
estrus
Natural estrus
-6 -5 -4 -3 -2 -1 1 2 3 4 5 6
FSH-P TREATMENT
DAY

Figure 7. PMSG-stimulated mature ovarian follicles 26 hours after adminstration of hCG.



cats demonstrating behavioral estrus, the uterine tone rating, the number of ovarian follicles available for aspiration, the number of oocytes collected or oocyte recovery efficiency (Table 5). The treatment interval/hCG interaction data (Table 6) indicated that the proportion of cats exhibiting overt estrus, the uterine morphology rating, the mean number of mature follicles and aspirated oocytes as well as the oocyte recovery rate also were not affected ($P>0.05$) by treatment. Frequently, more oocytes were detected in the aspirates then could be explained on the basis of the number of follicles aspirated (Table 6, 72 hour PMSG-hCG interval/100 IU hCG). Based on the degree of cumulus cell expansion, oocytes of varying maturational status were collected (Fig. 8), with less than 10% considered unsuitable for fertilization *in vitro* (Tables 5 and 6).

The ultrastructural morphology of oocytes subjected to fertilization, centrifugation and Hoescht staining is presented in Figure 9a. The mean diameter (n=17) of a mature domestic cat zona pellucida and oocyte combined was 162.4 ± 2.9 μ. The mean zona width was 17.4 ± 0.5 μ, and the mean oocyte diameter was 127.5 ± 2.9 μ. A germinal vesicle was not identifiable in an uncentrifuged oocyte but after high-speed centrifugation could be recognized as a membrane-bound structure comprising approximately 20% of the intercellular mass (Fig. 9b). Relative to the size of the entire oocyte, the polar bodies were relatively small (approximately 10 μ in diameter), visible only with DIC optics (Fig. 9c-e) and could by confirmed by Hoescht staining (Fig. 9f). Within a one-cell cat zygote, the pronuclei were bounded by well-defined membranes but contained no prominent nucleoli (Fig. 10a,b) so that distinguishing these organelles from intracellular vacuoles was difficult without DNA-specific staining (Fig. 10c,d). With fluorescence optics, these chromosomal structures were three-quarter moon-shaped and produced a "softer" brightness than somatic cell nuclei (as would be expected for a haploid structure). Although two-cell embryos were relatively simple to identify (Fig. 11a), at later developmental stages the compactness and opacity of the blastomeres made it difficult to distinguish between 4-cell to morula-stage embryos (Fig. 12a, 13a). Individual blastomeric nuclei were recognizable when embryos

Table 5. Estrous behavior, uterine tone and oocyte aspiration results of domestic cats by gonadotropin interval and hCG treatments

Treatment	No. of cats	Estrous behavior-number (%)	Mean uterine tone	Follicles aspirated number	Follicles aspirated mean	Oocytes collected number	Oocytes collected mean	% Recovery	No. immature [b] oocytes (%)
Gonadotropin Interval[a]									
72 hr	31	16 (52)	3.1±0.1	341	11.0±1.0	335	10.8± 1.1	98.2	24 (7.2)
80 hr	24	13 (54)	3.2±0.1	301	12.5±1.1	256	10.7± 0.9	85.0	23 (9.0)
hCG dose									
100 IU	25	13 (52)	3.1±0.1	298	11.9±1.1	295	11.8± 1.1	99.0	18 (6.1)
200 IU	30	16 (53)	3.2±0.1	344	11.5±1.0	296	9.9± 0.9	86.0	29 (9.8)

[a]Time interval between PMSG and hCG.
[b]Oocytes with tight corona radiata and cumulus cell mass.

Table 6. Estrous behavior, uterine tone and oocyte aspiration results from gonadotropin-treated domestic cats

Gonadotropin interval[a]/ hCG dose	No. of cats	Estrous behavior- number (%)	Mean uterine tone	Follicles aspirated number	Follicles aspirated mean	Oocytes collected number	Oocytes collected mean	% Recovery	No. immature[b] oocytes (%)
72 hr/100 IU	12	6 (50)	3.2 ±0.1	135	11.2 ±2.0	150	12.5 ±2.2	111.1	11 (7.3)
72 hr/200 IU	19	10 (53)	3.1 ±0.1	206	10.8 ±1.3	185	9.7 ±1.3	89.8	13 (7.0)
80 hr/100 IU	13	7 (54)	3.1 ±0.2	163	12.5 ±1.4	145	11.2 ±1.2	89.0	7 (5.0)
80 hr/200 IU	11	6 (54)	3.3 ±0.2	138	12.5 ±1.7	111	10.1 ±1.2	80.4	16 (8.6)

[a]Time interval between PMSG and hCG.
[b]Oocytes with tight corona radiata and cumulus cell mass.

Figure 8. Sequential morphological changes observed with increasing maturity of domestic cat oocytes: a) immature oocyte with a tightly compacted corona radiata, b) immature oocyte with a tightly compacted corona radiata and cumulus cell mass, c) cumulus cell mass demonstrating a slight degree of expansion and d) a mature oocyte demonstrating pronounced expansion of the corona radiata and cumulus cell mass.

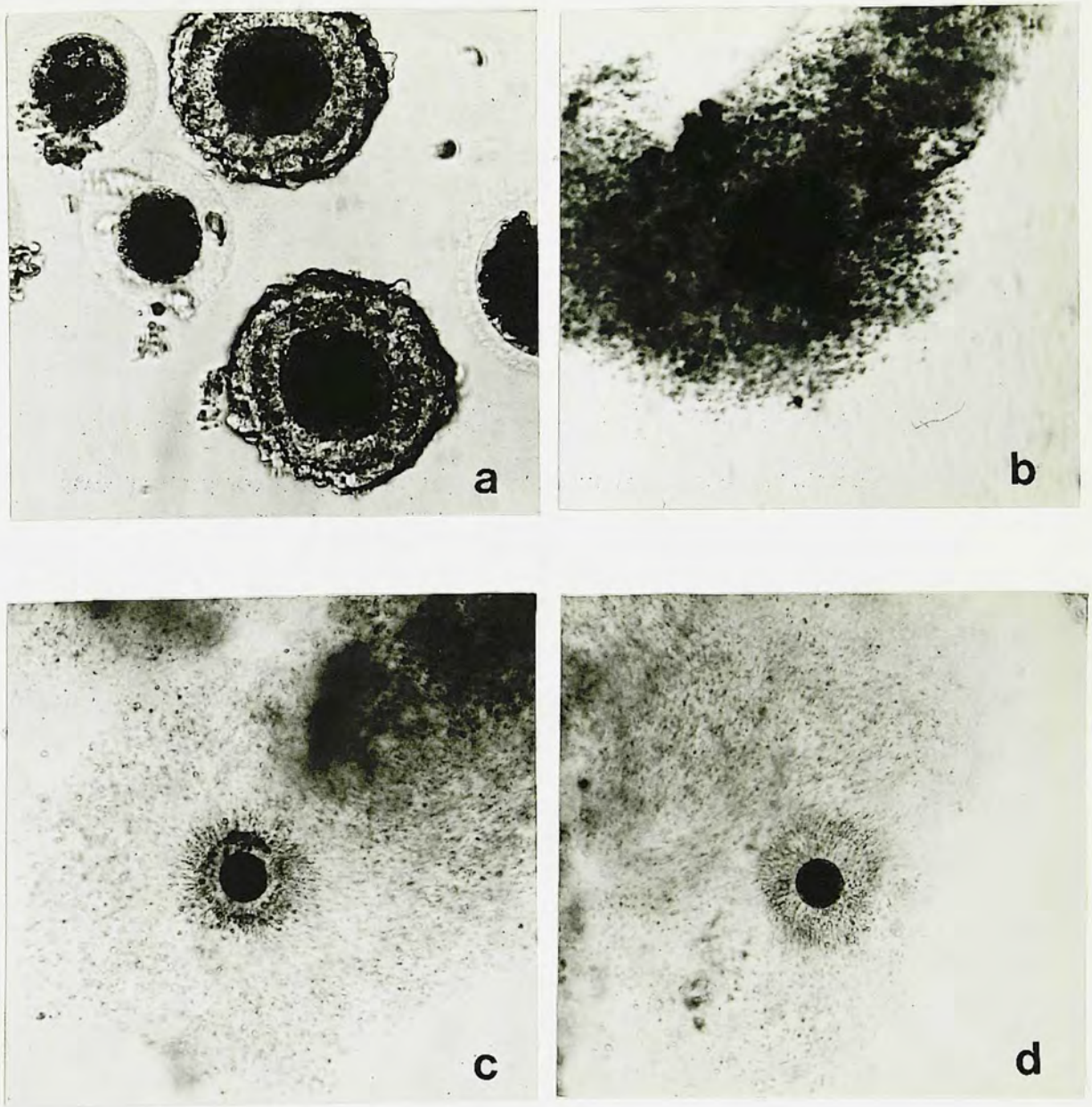

a
b
c
d

Figure 9. Morphology of unfertilized domestic cat oocytes, post-centrifugation (a) and demonstrating a germinal vesicle (b). Oocytes having undergone germinal vesicle breakdown as evidenced by a single polar body observed with DIC (c,d,e) or fluorescence optics (f) after Hoescht staining.

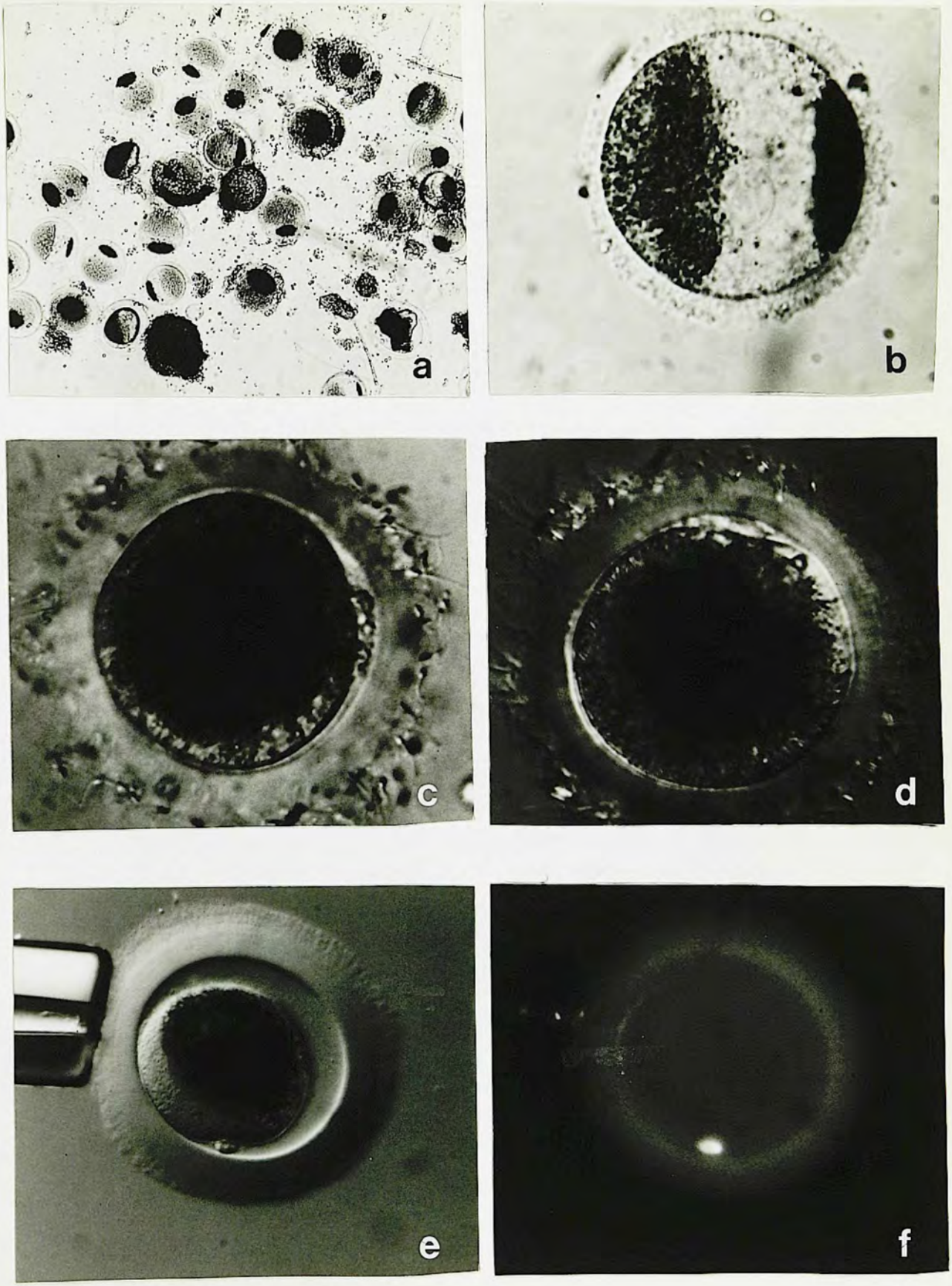

a
b
c
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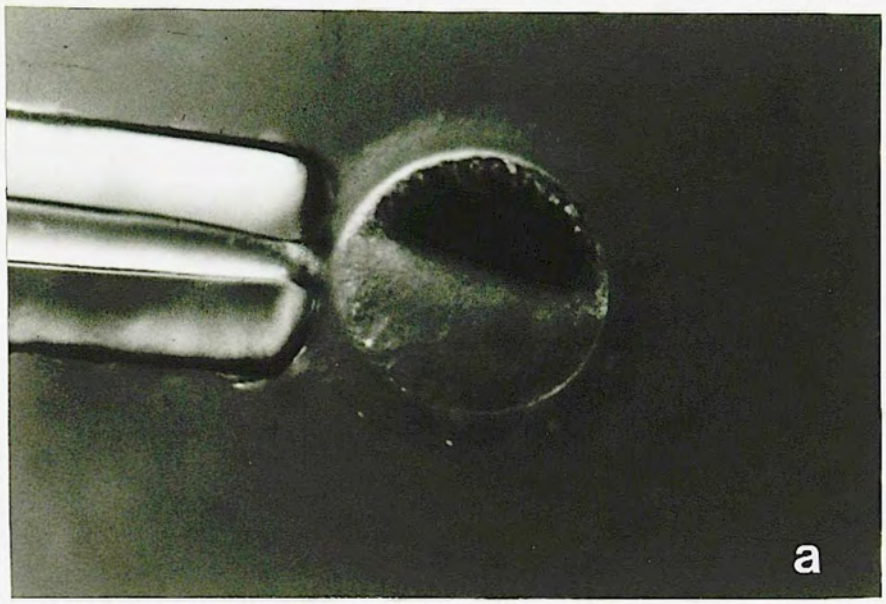
Figure 10. Centrifuged, one-cell *in vitro* fertilized, domestic cat oocytes as evidenced by the presence of two pronuclei with DIC (a) and fluoresence (b) optics following Hoescht staining.


a

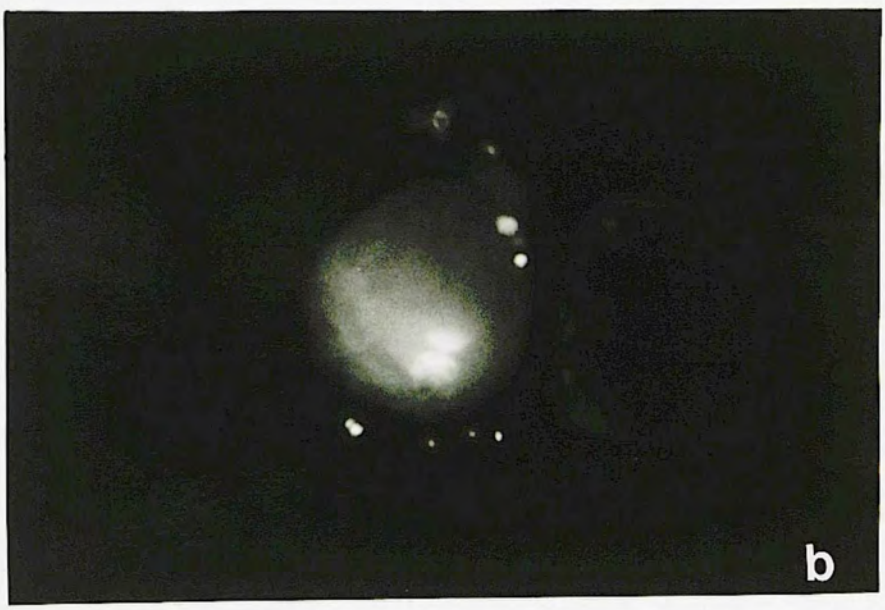
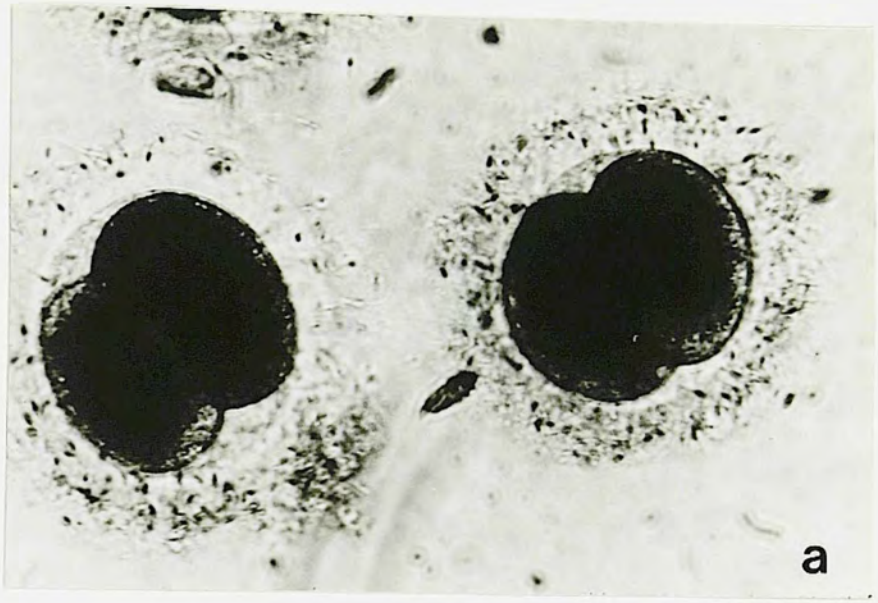

b

Figure 11. Two-cell stage *in vitro* fertilized domestic cat embyros observed with phase contrast optics (a). Two-cell (b) embryo subjected to Hoescht staining to observe individual nuclei.


a

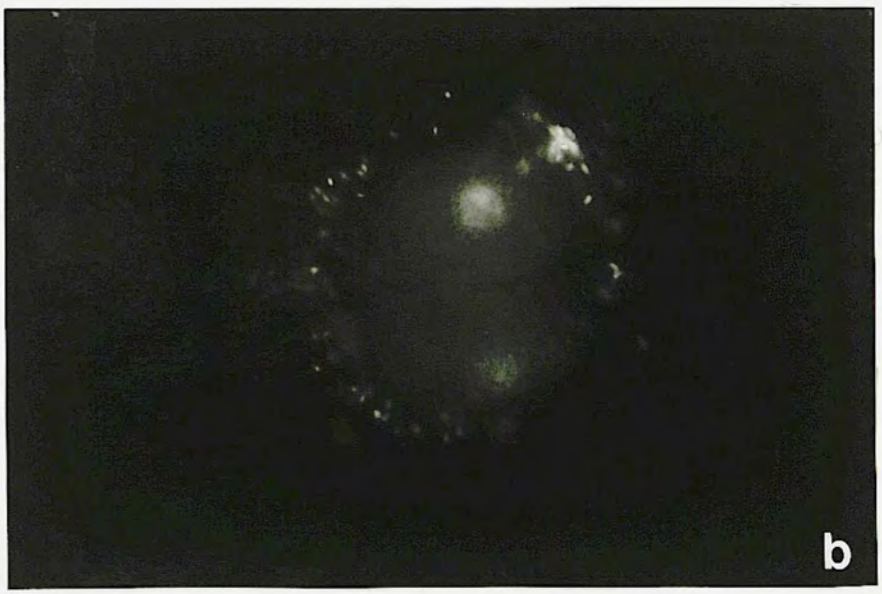
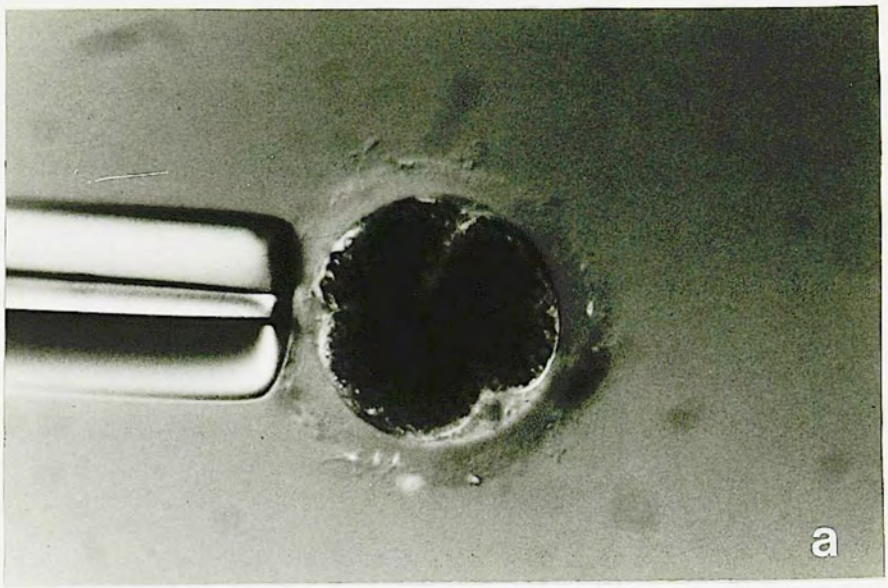

b

Figure 12. Four-cell stage *in vitro* fertilized domestic cat embryo observed with DIC optics (a). Four-cell stage embryo flattened and subjected to Hoescht staining to observe individual nuceli (b).


a

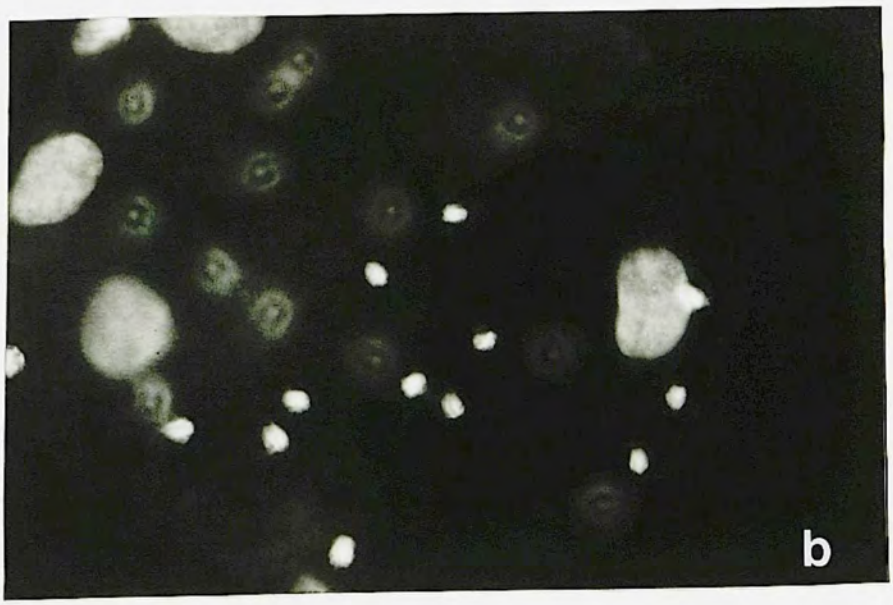
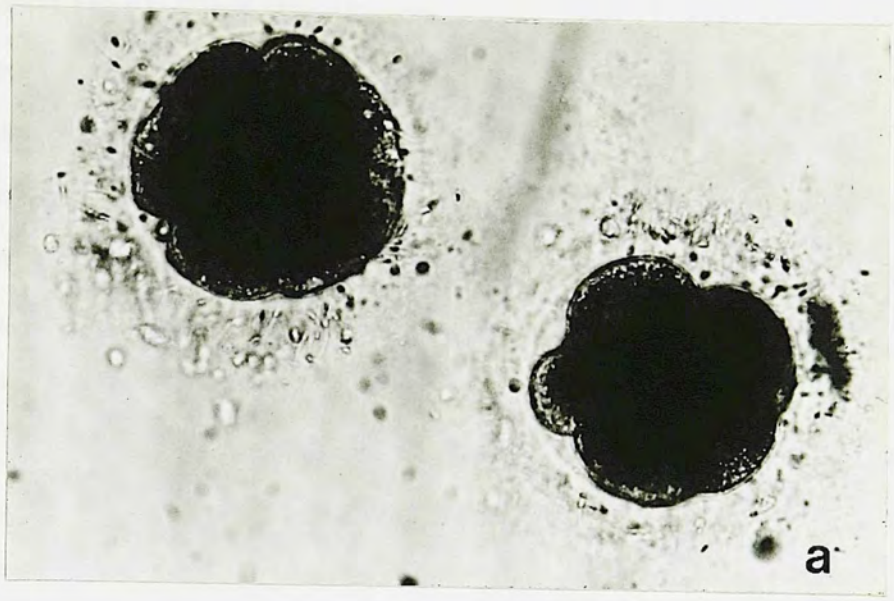

b

Figure 13. Morula-stage *in vitro* fertilized domestic cat embryos observed with phase contrast microscopy (a). Morula-stage embryo flattened and subjected to Hoescht staining to observe individual nuclei (b).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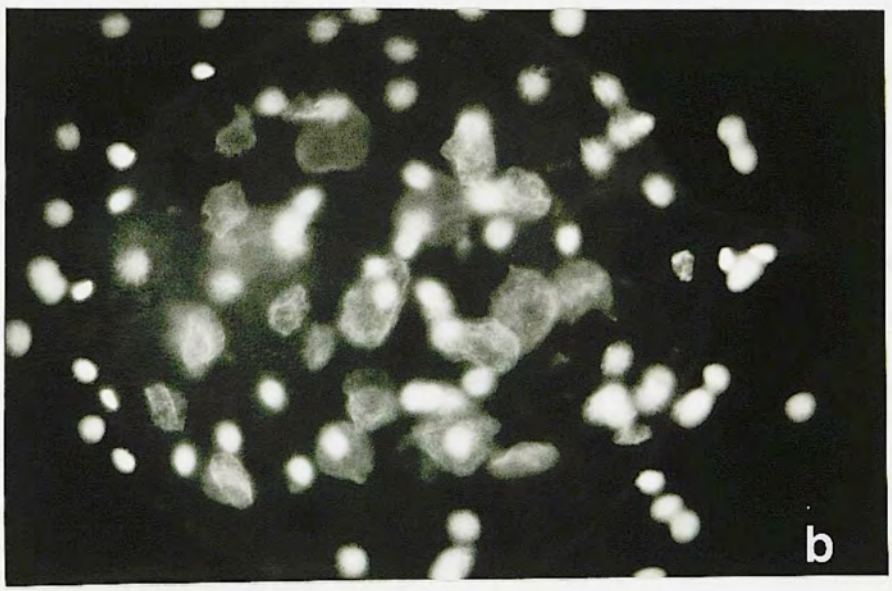

b

were Hoescht-stained, flattened under a coverslip and examined by fluorescence microscopy, thereby allowing an accurate count of cell number. (Fig. 11b, 12b, 13b). Within the two hormone interval and dose treatments (Table 7) or among the treatment interactions (Table 8), there were no differences ($P>0.05$) in the number of immature oocytes (those with germinal vesicles) or oocytes with one polar body only, one pronucleus only or one polar body plus one pronucleus. Approximately 10 to 20% of all oocytes had not experienced germinal vesicle breakdown when examined immediately post-collection. Overall, the number of polyspermic oocytes (defined as the number of oocytes with more than two pronuclei) was less than 4% and did not vary ($P>0.05$) by treatment (Table 7) or treatment interaction (Table 8). Although the hormone interval treatment had no influence on the number of degenerate ova, a greater proportion of the oocytes from cats treated with the 200 IU hCG dose appeared morphologically abnormal (Table 7, $P<0.001$), a finding which also was evident in examining the interaction results (Table 8). Likewise, although the proportion of one-cell embryos was unaffected by the various treatments, the number of cleaved ova was influenced by hormonal interval ($P<0.001$) as well as the hCG dose (Table 7, $P<0.025$). Extending the interval between PMSG and hCG by 8 hours doubled the cleavage rate whereas the lower dosage of hCG increased the cleavage rate by 10%. The overall percent fertilization rate was particularly sensitive to the PMSG/hCG interval with the proportion increased by at least 10% ($P<0.001$) in the 80 hour compared to the 72 hour group. Similarly, based on treatment interaction, the greatest ($P<0.05$) cleavage rate was observed with a combination of the 80 hour PMSG/hCG interval and the 100 IU hCG dose. Two of 32 control oocytes (6%) showed morphological evidence of parthenogenetic cleavage.

Embryos cultured in mKRB medium for 24 hours developed to the 4-cell stage while a 48 to 72 hour culture interval allowed development to at least the 16-cell stage, as evidenced by the presence of numerous blastomeres (Fig. 13a) and nuclei identified with Hoescht staining (Fig. 13b).

Table 7. Results from *in vitro* fertilization of domestic cat follicular oocytes by time interval or hCG dosage

Treatment	Total no. of oocytes	No. with germinal vesicles (%)[a]	No. with 1 pb, 1 pn or 1 pb/1 pn (%)[b]	No. polyspermic (%)	No. degenerate (%)[b]	No. of one-cell embryos (%)	No. of cleaved embryos (%)	Percent fertilized
Gonadotropin interval[c]								
72 hr	202	41 (20.3)	56 (27.7)	4 (2.0)	31 (15.4)	40 (19.8)	30 (14.8)[d]	34.6[d]
80 hr	214	30 (14.0)	51 (23.8)	7 (3.3)	29 (13.6)	37 (17.3)	60 (28.0)[e]	45.3[e]
hCG dose								
100 IU	207	37 (17.9)	60 (29.0)	4 (1.9)	17 (8.2)[d]	34 (16.4)	55 (26.6)[f]	42.0
200 IU	209	34 (16.3)	47 (22.5)	7 (3.4)	43 (20.6)[e]	43 (20.6)	35 (16.8)[g]	37.3

[a]Post-insemination.
[b]pb=polar body, pn=pronucleus.
[c]Time interval between PMSG and hCG.
[d,e]Values within columns and treatment with different superscripts differ, P<0.001.
[f,g]Values within columns and treatment with different superscripts differ, P<0.025.

Table 8. Results from *in vitro* fertilization of domestic cat follicular oocytes

Gonadotropin interval[a]/ hCG dose	Total no. of oocytes	No. with germinal vesicles (%)[b]	No. with 1 pb, 1 pn or 1 pb/1 pn(%)[c]	No. polyspermic (%)[b]	No. degenerate (%)	No.of one-cell embryos (%)	No. of cleaved embryos(%)	Percent fertilized
72 hr/ 100 IU	101	19 (18.8)	35 (34.6)	1 (1.0)	8 (7.9)[d]	21 (20.8)	17 (16.8)[f]	37.6
72 hr/ 200 IU	101	22 (21.8)	21 (20.8)	3 (3.0)	23 (22.8)[e]	19 (18.8)	13 (12.9)[f]	31.7
80 hr/ 100 IU	106	18 (17.0)	25 (23.6)	3 (2.8)	9 (8.5)[d]	13 (12.3)	38 (35.8)[g]	48.1
80 hr/ 200 IU	108	12 (11.1)	26 (24.1)	4 (3.7)	20 (18.5)[e]	24 (22.2)	22 (20.3)[f]	42.6

[a]Time interval between PMSG and hCG administration.
[b]Post-insemination.
[c]pb=polar body, pn=pronucleus.
[d,e]Values within columns with different superscripts differ, P<0.005.
[f,g] Values within columns with different superscripts differ, P<0.001.

Of the four queens receiving oviductal autotransfers of *in vitro* fertilized embryos, three were diagnosed pregnant at 35 days of gestation and eventually produced litters (Table 9, Fig. 14). In the first pregnancy, queen #1 experienced labor beginning 67 days after oocyte collection (66 days after oocyte cleavage *in vitro* and 65 days after autotransfer). After producing two kittens vaginally, uterine contractions inexplicably were arrested and the female did not respond to sequential oxytocin injections to induce further myometrial contractions. Therefore, the two remaining offspring were delivered by Caesarean section. At laparotomy, it was determined that one of these kittens was lodged cranially in the cervical os. Three of the four offspring survived, one dying as a result of compromised respiration, presumably contributed by anesthesia of the queen during the Caesarean section. Because of the prolonged gestation in queen #2, her healthy kitten was delivered by Caesarean section at 69 days (after oocyte collection) and immediately after the onset of labor. The pregnancy of queen #3 was allowed to continue to natural term and three of four offspring were born alive 67 days after oocyte collection; the last kitten delivered was stillborn. Another kitten had an umbilical hernia and died on the day of delivery. Each queen expressed normal maternal behavior and nursing capabilities and all surviving kittens developed normally through weaning at 8 weeks of age. The fourth queen receiving embryos did not exhibit obvious signs of pregnancy. Three and a half months after transfer, this female was diagnosed as having metastatic mesenteric lymphosarcoma and was euthanized.

Table 9. Embryo transfer results following *in vitro* fertilization of domestic cat follicular oocytes

Female	No. of follicles aspirated	No. of corpora lutea[a]	Date of transfer	No. of 2-4 cell embryos-transferred	Onset of labor	Duration of gestation[b]	No. of kittens (M/F)
1	6	6	1/29/87	18	4/4/87	67 days	4 (2/2)
2	24	20	1/29/87	13	4/6/87	69 days	1 (0/1)
3	10	10	2/5/87	8	4/11/87	67 days	3 (2/1)

[a]Observed at laparotomy.
[b]Number of days from oocyte collection to parturition.

Figure 14. Kittens resulting from *in vitro* fertilization of domestic cat follicular oocytes.

Assessment of corpus luteum formation and function after follicular aspiration

Two morphological types of CL could be distinguished in PMSG/hCG treated-cats following follicular aspiration. Type I CL were 3 to 6 mm in diameter, bright reddish-orange in coloration and raised prominently above the ovarian surface (in the shape of a mushroom, Fig. 15a). The coloring of the second type was a less intense, reddish orange. The size of these Type II CL ranged from 3 to 5 mm in diameter, and could be differentiated from Type I CL primarily on the basis of the flattened, more diffuse appearance (Fig. 15a,b). Naturally ovulating (NO) females demonstrated both CL types, with neither being predominant.

The mean number of pre-ovulatory follicles (2 to 5 mm in diameter) prior to aspiration and the mean number of CL 1 week after aspiration were similar between the PMSG/hCG treatment groups, but both were greater ($P<0.01$) than the respective values in the NO group (Table 10). In all but a single PMSG/hCG-treated queen, the number of CL exceeded the number of follicles identified and aspirated 1 week earlier (Table 10). In one cat, the number of CL exceeded the number of follicles aspirated by five-fold. In contrast, the number of CL in the NO queens corresponded to the number of mature follicles observed before mating. During the 8 week post-aspiration interval, CL number within individual queens of all groups remained constant while each individual CL gradually regressed to the corpus albicantia stage (Fig. 15c-e). In contrast to the ovarian response of FSH-P-treated females in Study 1, no cystic-appearing follicles were observed on the ovaries of any cat post-ovulation or post-aspiration.

Temporal estradiol-17ß and progesterone profiles for the NO animals and PMSG/hCG treated queens receiving either 100 or 200 IU hCG are depicted in Figures 16 and 17, respectively. Overall, estradiol-17ß profiles were not different ($P>0.05$) among groups from immediately post-ovulation through Day 44. There was a rise ($P<0.05$) in estradiol-17ß in NO females coincident with the end of the luteal phase (Fig. 16A, Day 46) which was not evident in the PMSG/hCG-treated cats. Among all groups, the area under

Table 10. Number of mature follicles and formation of CL in domestic cats following natural mating or follicular aspiration.

Treatment	Female	No. of [a] preovulatory follicles	No. of corpora [b] lutea formed
Control	1	3	3
	2	5	5
	3	4	4
	4	5	5
Mean ± S.E.M.		4.2 ± 0.5[c]	4.2 ± 0.5[c]
100 IU hCG	5	16	19
	6	15	29
	7	15	22
	8	4	7
	9	2	10
	10	10	15
Mean ± S.E.M.		10.3 ± 2.5[d]	17.0 ± 3.3[d]
200 IU hCG	11	15	28
	12	19	14
	13	9	18
	14	8	15
	15	20	43
Mean ± S.E.M.		14.2 ± 2.5[d]	23.6 ± 5.4[d]

[a]Follicles detected at laparoscopy prior to mating (natural estrus females) or aspiration (hCG-treated females).

[b]Detected at laparoscopy 1 week after mating or aspiration.

[c,d]Values within columns with different superscripts differ ($P<0.01$).

Figure 15. Type I and Type II corpora lutea observed at laparoscopy 1 week (a) and 2 weeks (b) after follicular aspiration. Regressing CL (c,d) 4 to 5 weeks after follicular aspiration. Inactive ovary (e) 6 weeks after follicular aspiration.

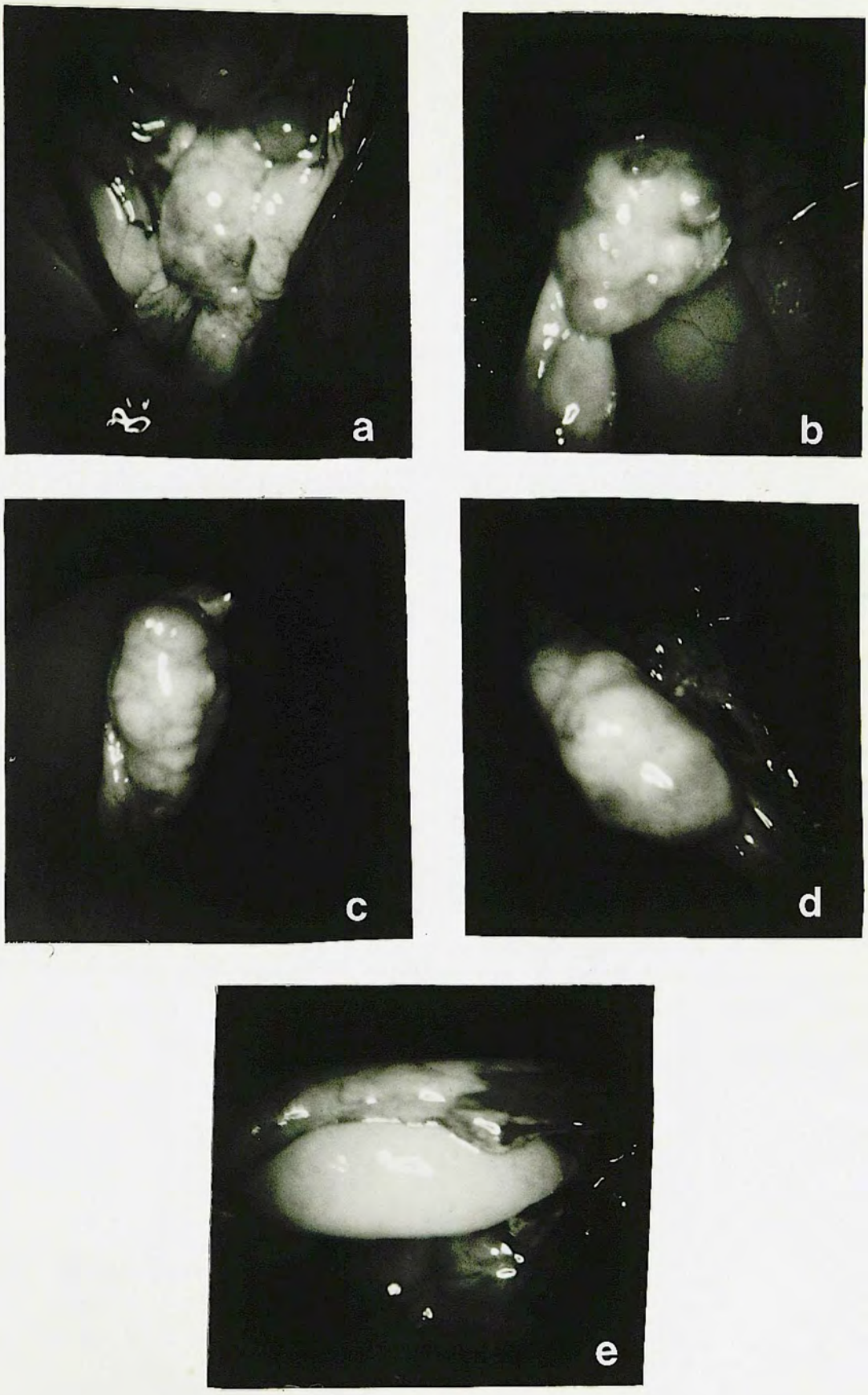

a
b
c
d
e

Figure 16. Mean (± S.E.M.) estradiol-17ß profiles during the 8 week sampling interval from natural estrus (control, panel A) cats or those treated with 100 (panel B) or 200 (panel C) IU hCG.

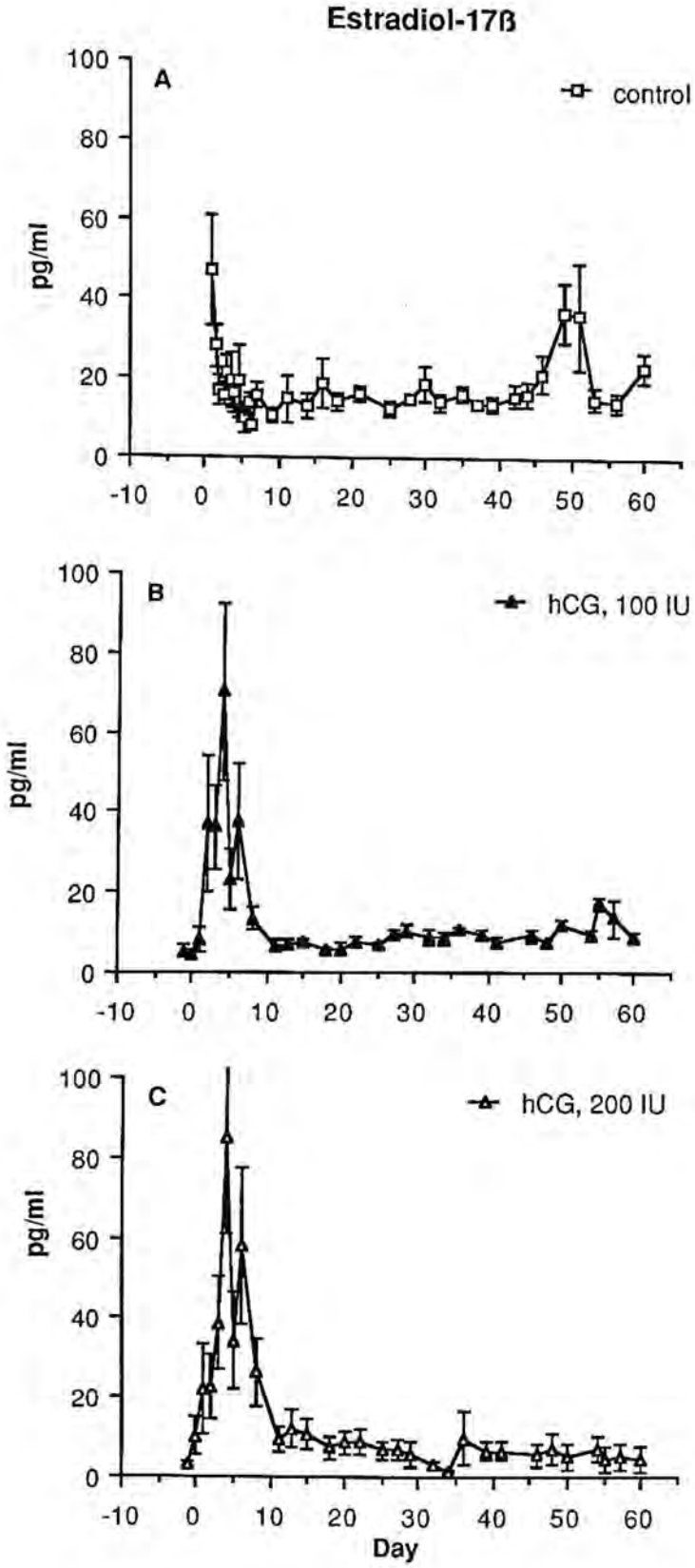
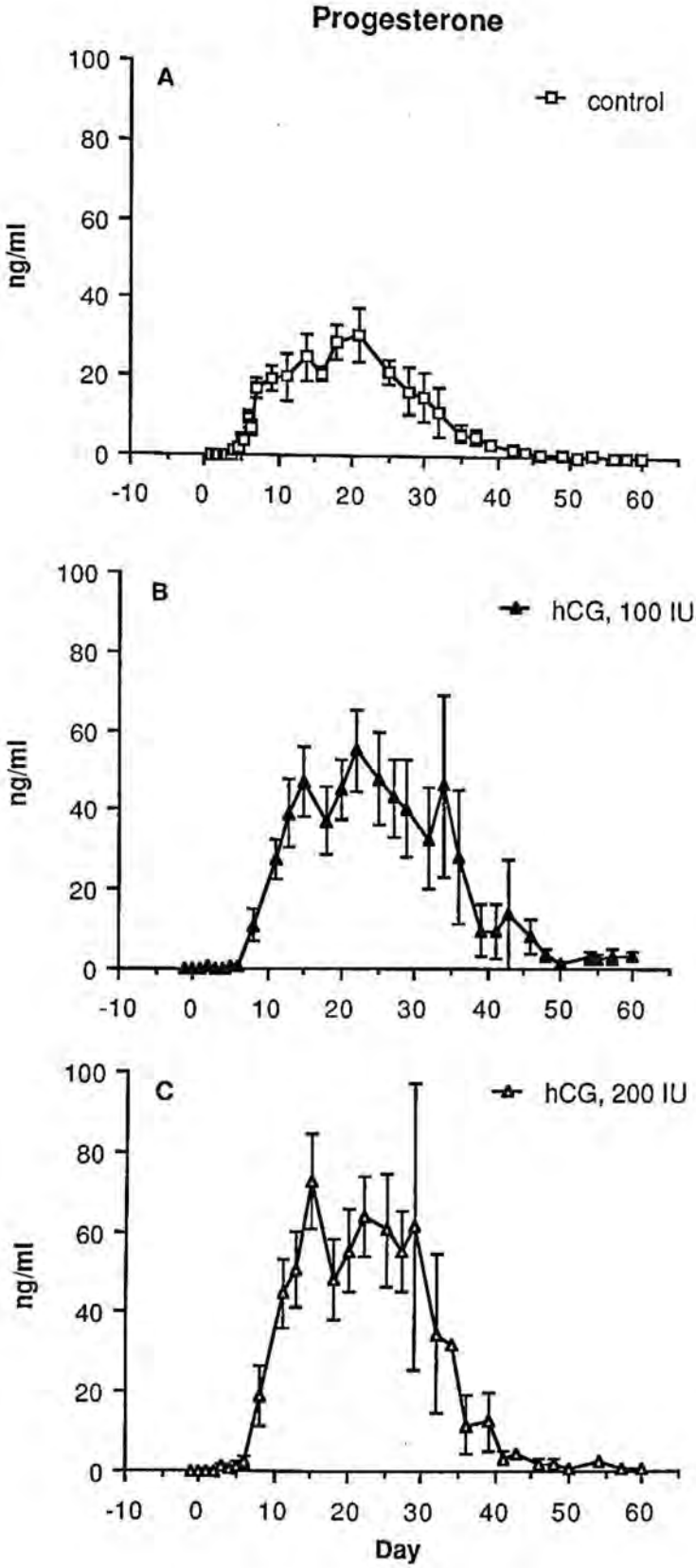

Estradiol-17ß
A
control
B
hCG, 100 IU
C
hCG, 200 IU
pg/ml
Day
100
80
60
40
20
0
-10
0
10
20
30
40
50
60

Figure 17. Mean (± S.E.M.) progesterone profiles during the 8 week sampling interval from natural estrus (control, panel A) cat or those treated with 100 (panel B) or 200 (panel C) IU hCG.


Progesterone
A
control
B
hCG, 100 IU
C
hCG, 200 IU
ng/ml
Day
100
80
60
40
20
0
-10
0
10
20
30
40
50
60

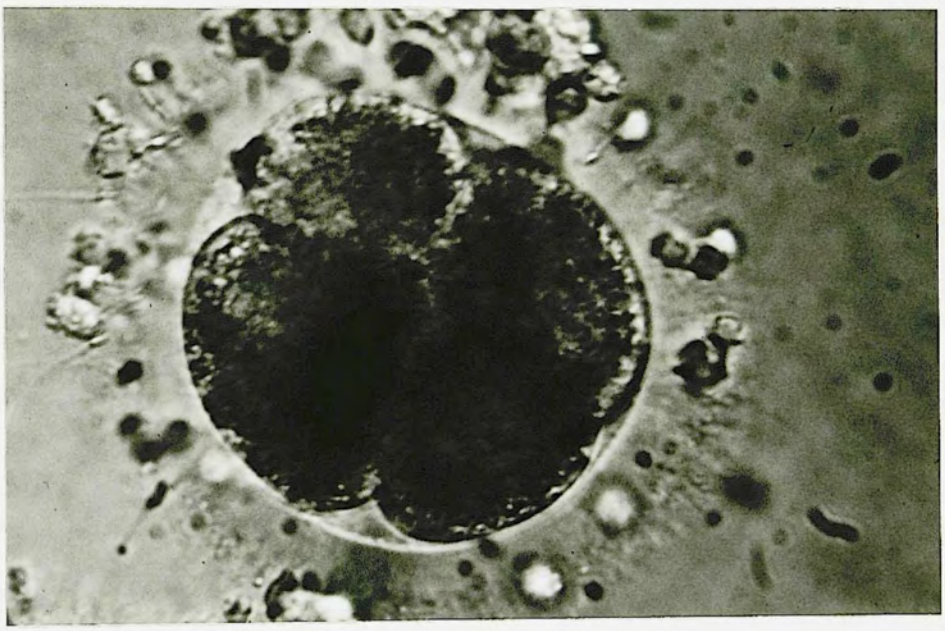
the progesterone profile curve was correlated positively with the number of CL ($r=0.78$, $P<0.001$). Although the mean areas under the curves did not differ ($P>0.05$) between the two hCG dosages (100 IU hCG, 14.2 ± 2.1 cm^2; 200 IU hCG, 18.1 ± 2.0 cm^2), the latter value was greater ($P<0.05$) than the mean area under the NO curve (8.5 ± 1.0 cm^2). The mean peak progesterone levels were similar (Fig. 17, $P>0.05$) between the 100 IU (55.1 ± 10.6) and 200 IU (72.4 ± 11.7) hCG groups, and although almost two-fold greater than the maximum progesterone concentrations in the NO group (30.8 ± 6.7), these values were not different. When the luteal phase was defined as the interval when circulating progesterone was greater than 1 ng/ml (Fig. 17), then the duration of this phase was similar ($P>0.05$) among all three groups.

In vitro fertilization of leopard cat oocytes

Female leopard cats responded to gonadotropin treatment with distinct ovarian follicle development following PMSG/hCG treatment. Overall, reproductive tract anatomy and ovarian changes over time simulated that observed in domestic queens. No evidence of estrual behavior was observed in any leopard cat following PMSG/hCG treatment, and on the day of follicular aspiration, the mean uterine tone rating was 2.3 ± 0.3. Following PMSG and 25 to 27 hours after hCG, slightly-raised ovarian follicles, 2 to 3 mm in diameter with distinct peripheral borders were observed. During 11 collection attempts, a total of 49 leopard cat follicles were aspirated resulting in the recovery of 47 oocytes (95.9% recovery). The mean number of follicles aspirated and oocytes recovered from leopard cats (4.4 ± 0.9 and 4.3 ± 1.2, respectively) were less ($P<0.01$) than the averages observed in comparably-treated domestic queens. Leopard cat oocytes were morphologically similar in size and appearance to domestic cat ova, being approximately 125 μ in diameter and having a homogeneously opaque appearance. Subjectively, leopard cat oocytes appeared to have fewer attached cumulus cells immediately post-aspiration compared to that observed for domestic cat ova. Twenty-six to 28 hours post-

insemination, 24 of the 47 oocytes collected (51.1%) were classified as degenerate. Eight ova contained germinal vesicles (17.2%), six had extruded a single polar body (12.7%), two demonstrated numerous pronuclei (polyspermy, 4.2%), and seven cleaved to at least the two-cell stage (14.9% fertilization rate, Fig. 18).

Figure 18. Four-cell embryo resulting from *in vitro* fertilization of laparoscopically collected leopard cat oocytes.



DISCUSSION

STUDY 1. FELINE EMBRYO COLLECTION AND TRANSFER

Reports of successful embryo transfer in carnivores are available for only three species. Chang (1968) produced one litter of ferrets after transferring morulae and blastocysts between two estrous females. The only report of embryo transfer in the dog was by Kraemer et al. (1979) who placed 37 embryos into seven bitches 9 to 10 days after the first mating; three pregnancies and three litters resulted. This same laboratory also produced the first offspring from domestic cats, transferring embryos between natural estrus donors and recipients (Kraemer et al., 1979). The present study demonstrates that successful embryo transfer is possible when ovarian activity and estrous behavior are induced with exogenous gonadotropins in both donor and recipient cats. However, the rate of success is relatively low and probably can be attributed to ancillary effects of the FSH-P treatment.

Domestic cats appear highly sensitive to exogenous gonadotropic preparations. Colby (1970) and Cline et al. (1980) administered PMSG and reported ovarian hyperstimulation including superovulation (as many as 60 CL/queen) and the formation of numerous cystic-appearing anovulatory follicles ($\geq$ 5 mm in diameter). Administering FSH-P for 5 consecutive days also can cause cystic, anovulatory follicle development (Wildt et al., 1978b; Goodrowe and Wildt, 1987), and, when FSH-P treated queens are artificially inseminated with fresh or frozen-thawed spermatozoa, low conception rates (~11%) result (Platz et al., 1978). In Study I, a high proportion of the cats given FSH-P and then mated (donors) or treated with hCG (recipients) ovulated, but also continued to maintain abnormal-appearing follicles during the early luteal phase.

The use of exogenous gonadotropins for ovulation induction has been associated with reduced *in vivo* and *in vitro* fertilization rates and poor early embryonic development

in the mouse, cat, rat and sheep (Fujimoto et al., 1974; Herron and Sis, 1974; Maudlin and Fraser, 1977; Evans and Armstrong, 1984a,b; Moor et al., 1985; Vanderhyden et al., 1986). These adverse effects have been attributed to the release of immature, atretic or chromosomally abnormal oocytes or altered oocyte transport. Any of these factors could be responsible for the differences in embryo quality and development observed, and all could have resulted directly from an altered endocrine milieu.

In Study I, the temporal and quantitative estradiol-17ß and progesterone patterns of NE cats were comparable to previous reports (Wildt et al., 1981a; Schmidt et al., 1983). Hormonally, queens responded rapidly to FSH-P-treatment, the first detectable rise in estradiol-17ß occurring within 12 hours of the initial injection. Subsequent elevations in estrogen were associated with repeated gonadotropin injections. However, the continuous rise of estradiol-17ß over the 5 day injection period could not be sustained into behavioral estrus, indicating that steroidogenesis within the FSH-P recruited follicles was prematurely altered. The FSH-P preparation apparently contains significant LH-like activity (Lindsell et al., 1986) which may have induced early follicular luteinization and ovulation thereby explaining the marked decline in circulating estradiol-17ß coincident with the premature rise in progesterone. Release of immature oocytes coupled with gamete aging and thus, asynchronous gamete interaction could have contributed to a reduced fertilization rate and poor embryo quality in the IE cats. In support of this hypothesis, Chang and Fernandez-Cano (1958) demonstrated that a prolonged interval between ovulation and sperm/oocyte interaction significantly decreased the fertilizability of ferret oocytes, while Marston and Chang (1964) observed that an increased time period between hCG and mating resulted in an elevated proportion of degenerate murine ova. The altered endocrine environment also could have affected sperm transport, as observed previously in ewes inseminated vaginally after FSH-P treatment (Evans and Armstrong, 1984b). Although not studied in most carnivores, sperm transport is known to be compromised in at least one species of wild felid (the tiger) after gonadotropin treatment. Flushing the reproductive tract of FSH-P-

treated tigers revealed no sperm cells in the oviduct or cranial aspect of the uterine horn 1 hour after vaginal insemination with fresh spermatozoa (Wildt et al.,1987).

It is clear that live-born domestic cat offspring can be produced from transferring embryos flushed from the reproductive tract after gonadotropin treatment of the donor. However, the hormone regimen used was not conducive to high pregnancy rates and a majority of the embryos from natural estrus queens did not produce term pregnancies, probably because recipients also were synchronized with FSH-P. Interestingly, other felids including the female cheetah and tiger also are responsive to FSH-P-treatment (Wildt et al., 1981b; Wildt et al., 1986b; 1987); however, insemination of FSH-P-treated females with freshly collected spermatozoa has failed to produce a single pregnancy. Before this technique can be applied to wildlife species, the surgical trauma associated with embryo recovery also must be addressed. Non-surgical embryo recovery will be a major prerequisite to the practical and repeatable use of embryo transfer in rare species where invasive surgery is contraindicated.

STUDY 2. *IN VITRO* FERITLIZATION OF FELINE OOCYTES

Although fertilization *in vitro* has been previously reported for the domestic cat (Hamner et al., 1970; Bowen, 1977; Niwa et al., 1985), the present data were the first which: 1) used gametes collected by a relatively atraumatic, practical approach; and 2) unequivocally demonstrated the developmental capacity of such embryos *in vivo*. Hamner et al. (1970), using ovulated oocytes flushed from the oviducts and spermatozoa capacitated within and retrieved from the uterus, reported that 44 of 92 oocytes (47.8%) appeared fertilized *in vitro* (range, 0 to 90%). Bowen (1977) also tested ovulated oocytes but with ductus deferens spermatozoa and two different media (mBSW, Ham's F-10) and reported fertilization rates of 77.8 and 80.1%, respectively. In this latter investigation, 102 oocytes were cultured with spermatozoa with the first evidence of embryo cleavage

observed 20 to 28 hours post-insemination, comparable to cleavage onset in the present study. To examine the events associated with early embryonic development , Niwa et al. (1985) examined spermatozoal penetration and pronuclear formation of *in vitro* fertilized cat oocytes. A total of 59 oviductal ova were observed from 15 minutes to 5 hours following insemination with epididymal spermatozoa. Penetration rates ranged from 0 to 100%, and decondensing sperm heads and male pronuclear formation were observed 3 to 4 hours after insemination. In contrast, pronuclear formation within follicular oocytes in this study occurred 20 to 22 hours after the addition of spermatozoa. The difference between studies likely was attributable to natural variations in the maturational status of ovulated versus follicular oocytes as well as to variations in the capacitation requirements of epididymal versus ejaculated spermatozoa.

Ejaculated spermatozoa in the rabbit, mouse, rat, hamster, guinea pig, cow, sheep and pig reportedly are more difficult to capacitate than epididymal or ductus deferens sperm cells (see reviews: Anderson, 1977; Brackett, 1981a; Yanagimachi, 1981), presumably because of a "decapacitation factor" in the seminal fluid. This factor has not been studied in cat seminal plasma, although Hamner et al. (1970) reported that ejaculated spermatozoa collected from domestic cats were incapable of fertilizing oocytes *in vitro* without prior *in utero* incubation. In the first of Hamner's three trials, spermatozoa incubated *in utero* for 30 minutes fertilized six of eight oocytes, while in the second and third trials, none of 15 inseminated oocytes cleaved. Uterine incubation of the sperm for 2 hours resulted in a 53% fertilization rate while a 24 hour residence resulted in 90% of the ova cleaving. Freshly ejaculated spermatozoa were incapable of penetrating the 22 oocytes tested. The present study demonstrates that electroejaculated cat spermatozoa subjected to mKRB medium and swim-up processing for 1 hour become capacitated *in vitro* without *in utero* exposure. The relative ease of spermatozoal capacitation varies significantly among individuals (Brackett, 1981a, Brackett, 1985) and appears species-specific; in certain animals successful capacitation requires specialized conditions such as a high ionic strength

medium (rabbit and bull, Brackett and Oliphant, 1975; Brackett, 1981a; Brackett et al., 1982), the addition of caffeine or cyclic AMP (Rhesus monkey, Boatman and Bavister, 1982) or simply slight variations in medium constituents (Rogers, 1978). Similar observations have been made in the dog, a carnivore species in which a slight modification of the sperm cell culture medium markedly influences *in vitro* penetration rates of homologous follicular ova (Mahi and Yanagimachi, 1976, 1978). However, because high rates of IVF were observed in three independent laboratories using four biologically different capacitation preparations (Hamner et al., 1970; Bowen, 1977; Niwa et al., 1985), domestic cat spermatozoa appear to be less sensitive to variations in preparation media. The ability to capacitate ejaculated domestic cat spermatozoa *in vitro* considerably increases the practicality of IVF as a tool for studying gamete interaction and improving reproductive efficiency.

Laparoscopy provided a consistently reliable and atraumatic approach for retrieving feline follicular oocytes, similar to that previously found in humans, monkeys and cattle (Steptoe and Edwards, 1978; Kuehl and Dukelow, 1979; Wood et al., 1981; Brackett et al., 1982; Lauritsen, 1983; Bavister, 1984; Balmaceda et al., 1984; Clayton and Kuehl, 1984; Sirad et al., 1985). However, compared to ovulated ova collected from the ovarian surface or oviducts, these follicular oocytes probably are less capable of fertilization and cleavage (Brackett, 1981a; Moor et al., 1983). In most instances, compared to ovulated ova, the intracellular protein and RNA content of follicular oocytes probably is insufficient for self-maintenance (Brackett, 1981a), and fewer of these cells have undergone germinal vesicle breakdown and polar body extrusion indicating incomplete nuclear maturation.

The germinal vesicles and pronuclei of ungulate oocytes (cow, Seidel, 1981; sheep, Boone et al., 1978) as well as those of laboratory animals (mice, McGrath and Solter, 1983; rats, Gaddum-Rosse et al., 1984) are readily identifiable. This morphological characteristic is of particular importance in assessing developmental stage as well as recognizing depot sites for injecting gene constructs associated with

micromanipulation or "genetic engineering" efforts. One distinct feature of all carnivore oocytes studied to date (ferret, dog, mink, cat) is the uniformly dark appearance of the oocyte and embryo (Chang, 1950, 1966, 1968; Chang and Yangimachi, 1963; Mahi and Yanagimachi, 1976, 1978; Bowen, 1977; Adams, 1981) which likely is attributable to a high intracellular lipid concentration (Guraya, 1965). This characteristic, which prevents determining meiotic status, also has been observed in oocytes and embryos of pigs (Wall et al., 1985). The high-speed centrifugation technique used by the latter investigators to circumvent this problem also was effective with domestic cat oocytes, readily displacing the intracellular lipid and allowing pronuclear identification.

In most common mammalian species, the extrusion of a polar body is easily identifiable (Brackett, 1981a, McGrath and Solter, 1983). In contrast, the polar bodies associated with cat oocyte maturation were relatively small in relationship to germ cell size, and for accurate recognition, required differential interference contrast optics and 25X magnification. Therefore, for the feline oocyte, polar body extrusion was not a readily effective criterion for judging maturational status. In the human, pig and mouse, the degree of corona radiata/cumulus mass expansion is the optimal indicator for oocyte maturity and has been positively correlated with fertilization *in vitro* (Gilula et al., 1978; Eppig, 1982; Brackett, 1985; Motlik et al., 1986). Because polar body extrusion was so difficult to identify in cat oocytes, cumulus cell mass expansion appeared to be the most consistently reliable index of maturation.

The success rate of IVF in the cat was highly dependent on the time interval between PMSG and hCG administration. Maintaining the oocytes intrafollicularly for 8 additional hours before the hCG challenge consistently facilitated fertilization including blastomere cleavage. The timing of gonadotropin therapy has been shown to influence oocyte retrieval, maturity and fertilizability *in vitro* for a variety of other species (Mizoguchi and Dukelow, 1980; Jones et al., 1983; Laufer et al., 1984; Hillier et al., 1985; Levran et al., 1985; Quigley et al., 1985; Ben-Rafael et al., 1986; Templeton et al., 1986), although

specific findings among different laboratories often are contradictory. In the human, excellent IVF rates were reported when the interval between the follicle stimulating gonadotropin (human menopausal gonadotropin, hMG) and hCG injection was 50 hours (Jones et al., 1983). However, Laufer et al. (1984) demonstrated that increasing the interval between hMG and hCG beyond 24 hours markedly increased the proportion of unfertilized and degenerate oocytes, presumably due to increased follicular atresia. More recent human studies have focused on the timing of hCG administration in relation to the endogenous rise in circulating estradiol concentrations. Quigley et al. (1985) and Levran et al. (1985) have demonstrated that a greater number of mature oocytes are recovered when hCG is given 6 days rather than either 5 or 7 days after the estradiol peak. Additionally, IVF and pregnancy rates after embryo transfer also were higher when this particular regimen was employed indicating that peak estradiol concentrations in the human is a useful index for ensuring the presence of mature, intrafollicular oocytes. In other species, varying the injection interval between PMSG and hCG also has been shown to affect IVF rates. When the administration interval between gonadotropins was increased from 56 to 72 hours in hamsters (Mizoguchi and Dukelow, 1980) and from 40 to 50 hours in mice (Hillier et al., 1985), fertilization rates increased significantly.

The present study demonstrated that hCG dose also influenced oocyte viability, fertilizability and developmental potential *in vitro*. Although greater hCG dosages have been shown to increase ovulation rates in the rabbit (Farrell et al., 1968), cat (Wildt and Seager, 1978) and hamster (Roldan et al., 1987), the two dosages tested in this study had no apparent effect on the number of mature follicles available for aspiration. Interestingly, the effect of therapeutic level of hCG on ovum integrity has not been addressed extensively in the literature, although there appears to be no detrimental effects of increasing hCG dosage on embryonic development in the hamster and mouse (Spindle and Goldstein, 1975; Roldan et al., 1987). In contrast, results from the present study indicated that a

greater hCG dose contributed to an increased incidence of oocyte degeneration and lower fertilization rates in culture.

Because the oocyte is incapable of independent metabolism, ovum maturation is dependent on cell-to-cell communication with the surrounding granulosa cells (by means of gap junctions) for the regulation of intercellular RNA, peptide synthesis, protein phosphorylation, and intercellular supplies of nucleotides, amino acids, enzymes and energy sources (Gilula et al., 1978; Moor et al., 1981; Downs et al., 1986; Motlik et al., 1986). The initiation of these final biochemical alterations are gonadotropin-stimulated, a follicle stimulating-type signal responsible for the final stages of oocyte maturation while an LH or hCG stimulus initiates nuclear maturation (Dekel at al., 1981; Eppig, 1982). Moor and Trounson (1977) demonstrated that, in the absence of gonadotropins, less than 5% of cultured/inseminated oocytes developed to the blastocyst stage while the addition of FSH and LH to the culture medium improved the developmental potential of cultured oocytes. However, it has been demonstrated that germinal vesicle breakdown and the metabolic events associated with cytoplasmic maturation are independent events, as evidenced by GVBD prior to cumulus cell mass expansion, and that nuclear maturation can occur within a metabolically immature oocyte (Moor et al., 1981; Eppig, 1982). Therefore, in the domestic cat, a poorly timed gonadotropin interval or an excessive ovulatory signal (hCG) may have resulted in premature granulosa cell uncoupling which interfered with final oocyte development and disrupted oocyte integrity and/or fertilizability. This hypothesis is supported by the data of Williams and Hodgen (1980) who reported that a premature hCG stimulus prohibits the granulosa cells from completing oocyte maturation in rhesus and cynomolgous monkeys. In contrast, prolonging the interval between sequential gonadotropin challenges can result in ova which fail to fertilize or that are vulnerable to polyspermy (Chang and Fernandez-Cano, 1958; Marston and Chang, 1964; Laufer et al., 1984; Quigely et al, 1985; Ben-Rafel et al., 1986). Because the cat is an induced ovulator, mature oocytes must be maintained within the follicle for unpredictable intervals following

estrus onset, in contrast to a fixed interval for most spontaneously ovulating species. Therefore, it is logical that an intrafollicular mechanism exists to maintain feline oocytes in an "ovulation ready" state for periods of 6 days or longer. Even so, the oocytes of this species remain highly sensitive to gonadotropin stimuli and apparently continue to experience critical maturational events up to the time of ovulation.

Aberrations such as parthenogenetic cleavage and polyspermic fertilization (Odor and Blandau, 1956; Yanagimachi and Chang, 1961; Marston and Chang, 1964; Kaufman, 1973; Maudlin and Fraser, 1977; Fraser and Maudlin, 1979; Longo, 1980), which occur because of alterations in oocyte integrity, can be influenced by gonadotropin stimulation and gamete aging. There is a wide variation in the incidence of parthenogenetic oocyte cleavage among species with "normal" values ranging from less than 5% for the rat (Austin, 1956) to as high as 60, 75 and 80% for the ferret (Chang, 1957), hamster (Austin, 1956; Chang and Fernandez-Cano, 1958; Yanagimachi and Chang, 1961) and pig (Dziuk, 1960), respectively. Parthenogenetic cleavage rates have not been reported previously for domestic cat oocytes, although to date, only ovulated-oviductal oocytes have been evaluated *in vitro* (Hamner et al., 1970; Bowen, 1977; Niwa et al., 1985). The present data demonstrate that follicular ova unexposed to spermatozoa are capable of spontaneously cleaving in culture, but the rate of parthenogenesis is relatively low (6%) and much less than the only other carnivore studied (the ferret, 80%, Chang, 1957). In certain strains of mice, parthenogenesis is a common event, and transfer of the resulting embryos has resulted in implantation and the development of primordial germ cells (Kaufman and Schnebelen, 1986). Although the birth of live young have been reported from embryos containing two female pronuclei (Hoppe and Illmense, 1977, 1982), it is now believed that both the maternal and paternal genome are necessary for full development (McGrath and Solter, 1983, 1984). Based on this information, it is highly unlikely that parthenogenesis can result in feline embryos capable of producing live offspring.

Although PMSG dose and sperm numbers *in vitro* have been positively correlated with increased proportions of polyspermic eggs (Maudlin and Fraser, 1977; Fraser and Maudlin, 1979), a more common cause appears to be oocyte aging (Odor and Blandau, 1956; Yanagimachi and Chang, 1961). As the interval after ovulation increased, Yanagimachi and Chang (1961) observed distinct changes in hamster ova indicative of degeneration including a decrease in the number of cortical granules at the cytoplasmic membrane and a weakening of the zona reaction, both resulting in increased polyspermia. Interestingly, Niwa et al. (1985) reported a relatively high rate of polyspermy (20%) in cat ova which was five times greater than the incidence observed in our study (4%). In contrast to follicular oocytes in this study, Niwa's oviductal ova were older, and therefore potentially more vulnerable to polyspermy. Additionally, these investigators used a higher dose of hCG and a longer PMSG to hCG interval which, based on the present results, appear to facilitate oocyte maturation as well as accelerate degeneration. The rate of polyspermic fertilization of follicular ova in our study was comparable to the 7% incidence reported for similarly collected human oocytes (Wentz et al., 1983; Englert et al., 1986) and did not appear to be influenced by gonadotropin interval or dose.

Sequential laparoscopic examinations in the domestic cat indicated that individual follicles punctured and aspirated underwent normal luteal development, and because progesterone levels rose over time, apparently normal steroidogenic changes. The progesterone profiles and luteal phase lengths were temporally similar to those of natural estrus cats mated with a vasectomized male and experiencing a "sterile" luteal phase (Wildt et al., 1981a). Similar findings have been observed in humans. Kerin et al. (1981) and Oskowitz et al. (1986) reported that in spontaneous cycles, aspiration of the single pre-ovulatory follicle following the LH surge resulted in progesterone profiles and luteal phase durations comparable to non-aspirated controls. Compared to untreated controls, domestic cats injected with PMSG/hCG quantitatively demonstrated greater progesterone production, which was positively correlated with corpora lutea number. In humans, treatment with

clomiphene citrate or hMG in combination with hCG results in elevated circulating progesterone concentrations (Kemeter et al., 1982; Dlugi et al., 1984; Huang et al., 1986; Vargyas et al., 1986) which has been attributed to multiple CL formation resulting from the gonadotropin therapy. A relationship between peripheral progesterone levels and number of CL is not unusual. As early as 1975, a correlation was known to exist between peak progesterone concentrations and CL number in the pig, a species normally producing 10 to 20 CL per estrous period (Webel et al., 1975). Although it appears likely that the punctured follicles were functionally competent and subsequently secreted progesterone, it is probable that some of the circulating progesterone was being contributed by "ancillary" CL which were noted at laparoscopy 1 week after oocyte aspiration. The reason for the formation of these CL is unknown, however at least one previous study has demonstrated that combined use of FSH-P and hCG in the domestic cat can result in delayed secondary follicle development and CL formation several days after ovulation (Goodrowe and Wildt, 1987). Perhaps a similar event occurred in the present study. Because of the long half-life of both of PMSG and hCG, it is possible that a prolonged effect of these gonadotropins occurred, resulting in further continued growth and ovulation following oocyte recovery. Accelerated and extreme elevations in circulating progesterone as a result of hormonal therapy have been shown to be detrimental in selected species. In the human, elevated progesterone secretion has been postulated to adversely affect the intrauterine environment, potentially contributing to failed implantation after embryo transfer (Garcia et al., 1981; Vargyas et al., 1986). Whether this occurs in the cat subjected to follicular aspiration is unknown because progesterone profiles were not monitored in the four queens subjected to embryo transfer. The high proportion of these cats becoming pregnant (three of four) would suggest that excessive progesterone production is not inhibitory to establishing a pregnancy; however, it is possible that the relatively small litter sizes compared to the number of embryos transferred could be explained on the basis of a partially compromised oviductal or uterine environment.

The leopard cat data are the first reported attempt to adapt laparoscopic oocyte recovery and IVF to a nondomestic felid species. The mechanical procedures, as developed for the domestic cat, were readily applied to the leopard cat which might be expected because of a similar body size and reproductive anatomy. However, there appeared to be species-specific differences between the two species in behavioral and ovarian responsiveness to the same exogenous PMSG/hCG regimen. Although the ovaries contained mature-appearing follicles 25 to 27 hours post-hCG, none of the leopard cats exhibited behavioral signs of estrus, likely due, in part, to their naturally aggressive temperament. The uterine tone rating of leopard cats more closely mimicked that of naturally estrus, mated domestic cats rather than domestic queens treated with the same gonadotropin regimen, suggesting that the endogenous endocrine environment of the leopard cat following gonadotropin stimulation was not excessively altered. There was a two-fold difference in the numbers of mature follicles available for aspiration and oocytes retrieved between the two species, with the leopard cat response more similar to that of a natural estrus domestic cat. Varying ovarian responses to different gonadotropic preparations are common among other species, including the sheep, cow and goat (Laster, 1973; Lauria et al., 1982; Armstrong and Evans, 1983; Armstrong et al., 1983), and have been associated with poor oocyte/embryo recovery and quality and pregnancy rates. Although *in vitro* fertilization of leopard cat oocytes resulted in embryo cleavage, the fertilization rate was low, due in part to a high incidence (51%) of degenerate oocytes. It appears that leopard cat oocytes are extremely sensitive to these specific gonadotropic preparations and/or dosages and that alterations in the hormonal treatment may be necessary to recover normal oocytes.

In summary, this project provides an overview of gamete physiology associated with fertilization and early embryonic development in the domestic cat. Although feline embryos can be successfully collected and transferred when ovarian activity is induced with exogenous FSH-P, the high proportion of unfertilized oocytes and poor quality embryos

collected from these cats suggests that fertilization and early embryonic development are impaired in FSH-P treated cats, perhaps as a result of alterations in serum estradiol-17ß and progesterone concentrations. Because of the hyperstimulatory effect of FSH-P and the trauma to the reproductive tract associated with embryo recovery, it appears that *in vitro* fertilization of PMSG-stimulated oocytes offers a more advantageous method for studying gamete interactions and embryology in felids. Domestic cat follicular oocytes are capable of becoming fertilized *in vitro*, but appear to be sensitive to alterations in the timing and dosage of gonadotropin adminstration, possibly because of alterations in intrafollicular maturation critical to oocyte integrity and maturational processes. The adaptation of the domestic cat IVF system to the leopard cat resulted in fertilization and cleavage of follicular oocytes. However, the high proportion of degenerate oocytes and low fertilization rates as compared to the domestic cat revealed striking species-specific ovarian and gamete responses to gonadotropin adminstration. These data clearly demonstrate that simple adaptation of such intricate procedures as *in vitro* fertilization to other Felidae species will not be possible without a preliminary, basic science approach directed towards establishing species-specific norms.

SUMMARY

Study 1. Embryo Collection and Transfer

1. Females treated with FSH-P produced more CL and unovulated follicles and a lower uterine tone than cats experiencing a natural estrus.
2. Although there were no differences in embryo recovery rates between the two groups, IE cats produced more developmentally retarded embryos, poor quality embryos and unfertilized oocytes than NE females.
3. Embryos collected from both NE and IE cats and transferred to IE recipients were capable of producing offspring.
4. Serum estradiol-17ß concentrations from IE cats were lower than NE cats on the first day of estrus.
5. Serum progesterone rose prematurely in IE cats when compared to NE females.

Study 2. *In vitro* fertilization of feline oocytes

1. PMSG-hCG interval, hCG dose and treatment interaction did not affect the proportion of cats exhibiting estrus, mean uterine tone, number of follicles aspirated, number of oocytes collected or percent oocyte recovery.
2. PMSG-hCG interval, hCG dose or treatment interaction did not influence the proportion of oocytes containing germinal vesicles, one polar body or exhibiting polyspermia.
3. Oocytes from females in the 80 hour PMSG-hCG group produced more cleaved embryos and a greater proportion fertilized than those in the 72 hour group.
4. Females receiving 100 IU hCG produced fewer degenerate oocytes and a greater proportion of cleaved embryos than those given 200 IU hCG.

5. Among treatments, females receiving 200 IU hCG produced more degenerate oocytes, regardless of the PMSG-hCG interval.
6. Females receiving 100 IU hCG 80 hours after PMSG produced a greater proportion of cleaved embryos and tended to produced more fertilized oocytes than any other treatment interaction.
7. *In vitro* fertilized embryos were developmentally competent and resulted in the birth of live-young following autotransfer.
8. Aspirated follicles developed into morphologically normal corpora lutea.
9. Serum estradiol-17ß profiles were similar among all groups during the 8 week bleeding period.
10. Serum progesterone profiles were temporally similar among PMSG-hCG treated and natural estrus cats, although PMSG treated females produced more progesterone which was correlated to corpora lutea number.
11. The IVF approach utilized in the domestic cat was adaptable to the leopard cat. However, after culture, leopard cat oocyte degeneration was more prevalent and the fertilization rate was lower compared to the domestic cat.

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APPENDIX I

Components of the modified Kreb's Ringer bicarbonate medium (Niwa et al., J. Reprod. Fertil., 74: 657-660, 1985)

Compound	mM
NaCl	94.60
KCl	4.78
$CaCl_2 \cdot 2H_2O$	1.71
KH_2PO_4	1.19
$MgSO_4 \cdot 7H_2O$	1.19
$NaHCO_3$	25.07
Na lactate	21.58
Na pyruvate	0.50
Glucose	5.56
BSA	4.0 g/l
Penicillin	0.05 g/l
Streptomycin sulfate	0.05 g/l

APPENDIX II

LIST OF COMMONLY USED ABBREVIATIONS

CL	corpus luteum/corpora lutea
FSH-P	follicle stimulating hormone
GVBD	germinal vesicle breakdown
hCG	human chorionic gonadotropin
IE	FSH-P induced estrus group, embryo transfer study
IVF	*in vitro* fertilization
LH	luteinizing hormone
mKRB	modified Kreb's Ringer bicarbonate medium
NE	natural estrus group, embryo transfer study
NO	naturally ovulating queens, IVF study
PMSG	pregnant mares' serum gonadotropin
QG	embryo quality grade
UFO	unfertilized ova
UT	uterine tone